PATENT COOPERATION TREA

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
WO 22973		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/IB 99/01553	17/09/1999	18/09/1998
Applicant		
LEK PHARMACEUTICAL AND CHEMICAL...et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[ ] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readble form.

[ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. ______

[ ] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[X] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB 99/01553

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07D309/30 C07D405/06 B01D15/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07D B01D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 92 16276 A (MERCK & CO INC) 1 October 1992 (1992-10-01) cited in the application the whole document ---	26-29 1-25
Y	US 4 231 938 A (MONAGHAN RICHARD L ET AL) 4 November 1980 (1980-11-04) see column 10 (isolation) ---	1-25
Y	EP 0 416 416 A (SCLAVO SPA) 13 March 1991 (1991-03-13) cited in the application page 1 ---	1-25
Y	US 5 427 686 A (ASHER WILLIAM J) 27 June 1995 (1995-06-27) column 6, line 3 - line 10 ---	1-25

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 November 1999	23/11/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Steendijk, M

1

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	FRENZ J: "FRONITERS OF BIPOLYMER PURIFICATION: DISPLACEMENT CHROMATOGRAPHY" LC-GC INTERNATIONAL (LIQUID AND GAS CHROMOTOGRAPHY),US,EUGENE, OR, vol. 5, no. 12, page 18-21 XP000350988 the whole document ---	1-25
Y	TINGYUE GU ET AL: "DISPLACEMENT EFFECT IN MULTICOMPONENT CHROMATOGRAPHY" AICHE JOURNAL,US,NEW YORK, NY, vol. 36, no. 8, page 1156-1162 XP000122652 ISSN: 0001-1541 the whole document -----	1-25

1

INTERNATIONAL SEARCH REPORT

ormation on patent family members

International Application No
PCT/IB 99/01553

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9216276 A	01-10-1992	US 5202029 A	13-04-1993
		AT 180986 T	15-06-1999
		CA 2104232 A	14-09-1992
		DE 69229393 D	15-07-1999
		EP 0578723 A	19-01-1994
		ES 2132121 T	16-08-1999
		JP 6506210 T	14-07-1994
US 4231938 A	04-11-1980	AR 224008 A	15-10-1981
		AT 2620 T	15-03-1983
		AU 535944 B	12-04-1984
		AU 5901780 A	18-12-1980
		BA 98222 B	02-08-1999
		BG 61205 B	28-02-1997
		CA 1161380 A	31-01-1984
		CS 221919 B	29-04-1983
		DD 157344 A	03-11-1982
		DK 254180 A,B,	16-12-1980
		EG 16957 A	30-06-1990
		EP 0022478 A	21-01-1981
		ES 492384 A	01-06-1981
		FI 801857 A,B,	16-12-1980
		GR 69216 A	07-05-1982
		IE 49685 B	27-11-1985
		IL 60219 A	31-05-1985
		JP 1518752 C	07-09-1989
		JP 57163374 A	07-10-1982
		JP 63066306 B	20-12-1988
		JP 1319910 C	29-05-1986
		JP 56008689 A	29-01-1981
		JP 58016875 B	02-04-1983
		KR 8302438 A	26-10-1983
		NO 801775 A	16-12-1980
		NZ 193922 A	25-05-1982
		PH 16913 A	12-04-1984
		PT 71371 A,B	01-07-1980
		RO 79487 A	06-07-1982
		SI 8011582 A	30-06-1997
		US 4294926 A	13-10-1981
		US 4319039 A	09-03-1982
		YU 152880 A	30-04-1983
		ZA 8003545 A	27-01-1982
		ZW 13480 A	03-02-1982
EP 0416416 A	13-03-1991	IT 1232311 B	28-01-1992
		AT 97911 T	15-12-1993
		CA 2024250 A	05-03-1991
		DE 69004900 D	13-01-1994
		DE 69004900 T	16-06-1994
		ES 2060881 T	01-12-1994
		JP 3118393 A	20-05-1991
		US 5043423 A	27-08-1991
US 5427686 A	27-06-1995	AT 182085 T	15-07-1999
		CA 2187090 A	12-10-1995
		DE 69510780 D	19-08-1999
		EP 0754083 A	22-01-1997
		JP 10501885 T	17-02-1998

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IB 99/01553

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5427686 A		WO 9526795 A	12-10-1995

Form PCT/ISA/210 (patent family annex) (July 1992)

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 12 May 2000 (12.05.00)	
International application No. PCT/IB99/01553	**Applicant's or agent's file reference** WO 22973
International filing date (day/month/year) 17 September 1999 (17.09.99)	**Priority date** (day/month/year) 18 September 1998 (18.09.98)
Applicant GRAHEK, Rok et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

17 April 2000 (17.04.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland** Facsimile No.: (41-22) 740.14.35	Authorized officer Pascal Piriou Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

RECEIVED
DEC - 1 2000
BROMBERG & SUNSTEIN

From the:
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

OSER Andreas
TIEDTKE-BÜHLING-KINNE
Bavariaring 4
D-80336 München
ALLEMAGNE

EINGEGANGEN
- 4. Juli 2000
TIEDTKE · BÜHLING · KINNE
PARTNER GbR

08 JAN 2001

PCT

WRITTEN OPINION

(PCT Rule 66)

Date of mailing (*day/month/year*)	03.07.2000
REPLY DUE	**within 3 month(s)** from the above date of mailing

Applicant's or agent's file reference WO 22973		
International application No. PCT/IB99/01553	International filing date (*day/month/year*) 17/09/1999	Priority date (*day/month/year*) 18/09/1998
International Patent Classification (IPC) or both national classification and IPC C07D309/30		
Applicant LEK PHARMACEUTICAL AND CHEMICAL...et al.		

1. This written opinion is the **first** drawn up by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

   - I ☒ Basis of the opinion
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain document cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

   When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

   How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

   Also: For an additional opportunity to submit amendments, see Rule 66.4.
   For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis.
   For an informal communication with the examiner, see Rule 66.6.

   If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 18/01/2001.

WV 3.8.

Frist : 3.10.00 nicht verlängerbar
Term : not extendible

Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer / Examiner Steendijk, M Formalities officer (incl. extension of time limits) Brell, S Telephone No. +49 89 2399 7271

I. Basis of th opinion

1. This opinion has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".*):

Description, pages:

1-15 as originally filed

Claims, No.:

1-29 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. This opinion has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	26-29
Inventive step (IS)	Claims	1-29
Industrial applicability (IA)	Claims	

2. Citations and explanations

see separate sheet

VIII. Certain observations on the int rnational application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

1) The present application relates to the purification of HMG-CoA reductase inhibitors involving the use of displacement chromatography (claims 1-25) as well as thus purified preparations of a purity exceeding 99.7%.

2) Reference is made to the following documents:

D1: WO-A-92 16276
D2: US-A-4 231 938
D3: EP-A-0 416 416
D4: US-A-5 427 686
D5: LC-GC International, 5(1992) No. 12, 18-21 (Hancock et al.)
D6: Aiche Journal, 36 (1990) No. 8, 1156-1161 (Gu et al.)

3) Novelty

The subject-matter of claims 26-29 is not novel over for instance document D1, which already discloses similar preparations of high purity. In this context it is noted that the method of purification is not considered to further characterize the purified product.

With respect to claims 1-25 it is observed that these claims involve displacement chromatography as purification method. Such displacement chromatography step is considered to comprise the introduction of a special displacer.
No available prior art seems to describe such a method for the purification of HMG-CoA reductase inhibitors:
documents D1 and D2 describe HPLC and elution chromatography for purification of HMG-CoA reductase inhibitors, not specifically the use of a special displacer; documents D3-D6 describe displacement chromatography applications, but not specifically for HMG-CoA reductase inhibitors.

In this context it is however noted that according to document D6 the displacement effect will occur in preparative chromatography in general; accordingly, in as far as claims 1-3 could be interpreted as including sample displacement chromatography, such subject-matter would not seem novel over D1 and D2.

4) Inventive step

Document D1 already proposed HPLC as method for HMG-CoA inhibitor purification. The problem underlying the present application in view of D1 seems the provision of a (possibly more) convenient purification method for HMG-CoA inhibitors resulting in high purity products.
As solution to such a problem the claimed subject-matter would seem obvious in view of any of documents D3-D6, which describe the various advantages of the displacement mode in preparative chromatography.

Should the applicant be able to argue convincingly that the teaching in the present application allows the non-obvious solution of some particular problem, it would appear that such an argument could only be valid for very specific subject-matter (compounds of specific structure to be purified, defined displacers to be used).

5) Further observations

The definition of the stationary phase as C-18 or C-8 (claim 17) seems unclear.
It would appear that the reference to US-A-5043432 on page 4 should actually read US-A-5043423.

PATENT COOPERATION TREATY

15

REC'D 13 OCT 2000
WIPO PCT

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference WO 22973	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/IB99/01553	International filing date *(day/month/year)* 17/09/1999	Priority date *(day/month/year)* 18/09/1998
International Patent Classification (IPC) or national classification and IPC C07D309/30		
Applicant LEK PHARMACEUTICAL AND CHEMICAL...et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 5 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand 17/04/2000	Date of completion of this report 11.10.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Steendijk, M Telephone No. +49 89 2399 8460

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IB99/01553

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-3,5-15	as originally filed			
4	as received on	25/09/2000	with letter of	25/09/2000

Claims, No.:

1-25	as received on	25/09/2000	with letter of	25/09/2000

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-25
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-25
Industrial applicability (IA)	Yes:	Claims	1-25
	No:	Claims	

2. Citations and explanations

see separate sheet

1) The present application relates to the purification of HMG-CoA reductase inhibitors involving the use of displacement chromatography (claims 1-25) which may allow the direct obtention of preparations of a purity exceeding 99.7%.

2) Reference is made to the following documents:

D1: WO-A-92 16276
D2: US-A-4 231 938
D3: EP-A-0 416 416
D4: US-A-5 427 686
D5: LC-GC International, 5(1992) No. 12, 18-21 (Hancock et al.)
D6: Aiche Journal, 36 (1990) No. 8, 1156-1161 (Gu et al.)

3) Novelty
Claims 1-25 relate to the application of displacement chromatography as purification method for HMG-CoA reductase inhibitors. Such displacement chromatography step comprises the introduction of a special displacer as particularly defined in the present claim 1.
No available prior art seems to describe such a method for the purification of HMG-CoA reductase inhibitors:
documents D1 and D2 describe HPLC and elution chromatography for purification of HMG-CoA reductase inhibitors, not specifically the use of a special displacer; documents D3-D6 describe displacement chromatography applications, but not specifically for HMG-CoA reductase inhibitors.

4) Inventive step
Document D1 already proposed HPLC as method for HMG-CoA inhibitor purification. The problem underlying the present application in view of D1 seems the provision of a (possibly more) convenient purification method for HMG-CoA inhibitors resulting in high purity products. In this context it is noted that the application shows that displacement chromatography may indeed allow the direct obtention of preparations of HMG-CoA reductase inhibitors of a purity exceeding 99.7%.

In as far as the displacement mode defined in the claim indeed results in the direct

preparation of HMG-CoA in a purity exceeding 99.7%, the subject-matter would not seem obvious to the person skilled in the art:
- although documents D3-D6 already mentioned various advantages of the displacement mode in preparative chromatography, document D5 actually mentioning the advantage of high resolution (compared with step elution) in the context of isolation of biopharmaceutical products of a wide variety of structures, such a purity, which is comparable or possibly exceeding the purity of preparations obtainable by HPLC+crystallisation as described in D1, seems extraordinary and could not have been be expected.

However, the claims would seem to cover any method for obtaining HMG-CoA reductase inhibitors involving a displacement chromatography step regardless of the purity resulting from such a chromatography step. Although the examples show that the purity exceeding 99.7% may indeed be directly obtained under a variety of conditions, it seems quite unlikely that any method involving displacement chromatography would directly result in such high purity.
In as far as the mentioned high purity is not directly obtained, no basis for an inventive step can be recognized considering that in view of the mentioned prior art (D3-D6, in particular D5) the person skilled in the art would regard displacement chromatography per se as an attractive purification method in particular for biopharmaceuticals and thus prima facie obvious as convenient step in the purification of HMG-CoA reductase inhibitors.
In this context it is noted, that in view of the clear recomendations presented in particular in document D5 the possible prior "mistery" or "aura of complexity" of displacement chromatography (also noted in D5) is not considered support any prejudice against such dispacement chromatorgraphy after the publication of D5. Similarly, the time lapsing (ca. 25 years) after on the one hand the first report of displacement chromatography (1943) and the introduction of the first HMG-CoA reductase inhibitors (1975) and on the other hand the filing of the present application is not considered as indication for an inventive step in view of the renewed interest in replacement chromatography reported in D5 (1992).

In view of the above, the contribution over the prior art that can be identified in the present application (cf. present claim 25 in relation with the examples) seems to reside in the recognition that displacement chromatography unexpectedly allows

for the possibility to directly obtain HMG-CoA reductase inhibitors in a purity of 99.7%. In view of this contribution over the prior art, an inventive step could be recognized for a modified claim 1 which defines that the displacement chromatography produces a HMG-CoA reductase inhibitor with HPLC purity exceeding 99.7% (cf. present claim 25).

PATENT COOPERATION TREATY

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DEC - 1 2000

BROMBERG & SUNSTEIN

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

PTO/PCT Rec'd 08 JAN 2001

To:

OSER Andreas
TIEDTKE-BÜHLING-KINNE
Bavariaring 4
D-80336 München
ALLEMAGNE

EINGEGANGEN
Patentanwälte
12. Okt. 2000
TIEDTKE · BÜHLING · KINNE
& PARTNER (GbR)

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

	Date of mailing *(day/month/year)* 11.10.2000	
Applicant's or agent's file reference WO 22973	**IMPORTANT NOTIFICATION**	
International application No. PCT/IB99/01553	International filing date *(day/month/year)* 17/09/1999	Priority date *(day/month/year)* 18/09/1998
Applicant LEK PHARMACEUTICAL AND CHEMICAL...et al.		

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

   The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

   Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

   For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Brell, S Tel.+49 89 2399-7271

Form PCT/IPEA/416 (July 1992)

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BROMBERG & SUNSTEIN
528 Rec'd PCT/PTO 03 JAN 2001

TBK

TIEDTKE - BÜHLING - KINNE & PARTNER (GbR)

TBK-Patent POB 20 19 18 80019 München

Patentanwälte
Dipl.-Ing. Harro Tiedtke
Dipl.-Chem. Gerhard Bühling
Dipl.-Ing. Reinhard Kinne
Dipl.-Ing. Hans-Bernd Pellmann
Dipl.-Ing. Klaus Grams
Dipl.-Ing. Aurel Vollnhals
Dipl.-Ing. Thomas J.A. Leson
Dipl.-Ing. Dr. Georgi Chivarov
Dipl.-Ing. Matthias Grill
Dipl.-Ing. Hans-Ludwig Trösch
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Dipl.-Ing. Rainer Böckelen
Dipl.-Ing. Jürgen Feldmeier
Dipl.-Ing. Stefan Klingele
Dipl.-Chem. Stefan Bühling
Dipl.-Ing. Ronald Roth

An das
Europäische Patentamt

80298 München

September 25, 2000

PCT Patent Application No.: PCT/IB99/01553
(F: 3.10. Eing.)
LEK PHARMACEUTICAL AND CHEMICAL ... et al.
Our ref.: WO 22973

This is in reply to the written opinion pursuant to Rule 66 PCT dated July 3, 2000.

1. Enclosed are filed specification replacement pages 4 (relating to the description) and 16 to 19 (relating to the claims).
Further examination shall be based on these documents together with the remaining part of the original specification.

2. Amendments

The subject-matter of claim 1 has been amended by specifying that the process involves the use of a displacer for displacing the HMG-CoA reductase inhibitor. Such use of a displacer is a characteristic of the displacement chromatography, as can be gathered, for example, from the original description on p. 3, l. 10-14.

Claims 2 to 25 of the new claim version remained unchanged.

Deutsche Bank	München	Kto. 286 1060	BLZ 700 700 10
Dresdner Bank	München	Kto. 3939 844	BLZ 700 800 00
Postbank	München	Kto. 67043 804	BLZ 700 100 80
Dai-Ichi-Kangyo Bank	München	Kto. 51 042	BLZ 700 207 00
Sanwa Bank	Düsseldorf	Kto. 500 047	BLZ 301 307 00

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Without prejudice, original claims 26 to 29 directed to the HMG-CoA reductase inhibitor product as such were removed from the claim version.

Furthermore, thanks to the Examiner's note under item 5 of the separate sheet of the written opinion, the US document cited on p. 4, l. 10 was corrected to read " US Pat. No. 5,043,423".

3. Novelty

3.1 In view of the omission of the original claims 26 to 29 from the present claim version, a corresponding objection is obsolete.

3.2 With respect to claim 1, the Examiner's observations have been taken into account and an appropriate clarification has been included in the wording of claim 1 by amendment in order to state a proper delimitation over the prior art of WO-A-92 16 276 (D1) and US-A-4 231 938 (D2).

Specifically, documents D1 and D2 respectively describe HPLC and elusion chromatography methods for the purification of HMG-CoA reductase inhibitors. Even if speculating that some "displacement effect" may occur as theoretically considered in D6, conventional chromatography by HPLC or elusion does definitely not involve the use of a displacer, which is typical for displacement chromatography. As discussed in D6, a theoretical "displacement effect" on the purification of a specific component of a sample may be based on the influence of another sample component. Especially when too large sample amounts are applied on the column, it may be possible that one sample compound can displace others on the stationary phase. However, this can be considered only as an undesired side effect, but is not

the principle on which elusion chromatography is based. Rather, the basis for elusion chromatography is the difference between distribution coefficients of the respective compounds in stationary and mobile phase. By contrast, the basis for displacement chromatography is the displacing of compounds from the stationary phase by the deliberate use of a displacer; the ratio between distribution effects as in conventional elusion chromatography is not of concern here.

Accordingly, it is believed that the amended version of claim 1 makes this distinction of displacement chromatography versus conventional HPLC or elusion chromatography more clear.

3.3 The remaining documents, i.e. EP-A-0 416 416 (D3), US-A-5 427 686 (D4), LC-GC International, 5 (1992) No. 12, pp. 18-21 (Hancock et al.) (D5) and Aiche Journal, 36 (1990) No. 8, pp. 1156-1161 (Gu et al.) (D6), all relate to displacement chromatography applications (D3 - D5) or to displacement effects in multicomponent chromatography (D6), but do not disclose the application of displacement chromatography for the purification of HMG-CoA reductase inhibitors.

3.4 Hence, the presently claimed process is novel over the cited prior art.

4. Inventive Step

4.1 Documents D1 and D2 represent the pertinent prior art relating to the purification of HMG-CoA reductase inhibitors. As can be seen from document D2 (see especially Example 6, description of the isolation process in col. 10 and 11), the isolation and purification of a HMG-CoA re-

ductase inhibitor (designated MSD 803) usually involves repeated elusion chromatography, gel permeation chromatography, crystallization and multiple re-crystallization steps.

The teaching of D1 proposes an improvement by the use of preparative HPLC chromatography to obtain a purity of at least 99.5 %. This HPLC process offers the advantage that no re-crystallization is required; however, crystallization is still necessary (see p. 2, l. 25-29 of D1). The examples of D1 show where already pre-isolated and pre-purified HMG-CoA reductase inhibitors are subjected to HPLC chromatography and are further purified by crystallization, achieving purity levels between 99.5 and 99.8 %.

4.2 Compared with D1 as the closest prior art, the objective problem is to be seen in providing an **improved** process for obtaining HMG-CoA reductase inhibitors in terms of process economy, process efficiency **and** product purity. The present specification demonstrates that this problem is actually solved.

4.3 It is submitted that the presently claimed subject-matter is not rendered obvious to the skilled person, who starts from D1 and faces the afore-mentioned problem, in view of any one of documents D3 to D6 which relate to various aspects of the displacement mode in preparative chromatography. This will become apparent from the following discussion.

4.4 It should be emphasized first that purity is the most important criterion when dealing with HMG-CoA reductase inhibitors which shall be effective for medical treatment. In particular, since HMG-CoA reductase inhibitor containing pharmaceutical products are frequently taken on a long-term

basis for the treatment or prevention of high plasma cholesterol levels, impurities in the pharmaceutical product of lower purity may accumulate during this treatment and may thereby cause serious side effects (see p. 1, 2nd paragraph of the text of the present application).

In view of this essential criterion, it should be apparent that the skilled person would not contemplate a purification technique which is basically different from the pertinent prior art (cf. D1 and D2), if a purity level at least as good as conventionally achieved in the art of HMG-CoA reductase inhibitors (see e.g. D1) could not be expected. This holds true even if a possibly more convenient purification method may thereby be applicable.

4.5 Referring to the documents dealing with displacement chromatography or displacement effects, the following is noted.

4.5.1 Document D6, strictly spoken, does not relate to displacement chromatography within the concept of the present invention, i.e. intentionally using a displacer compound. Rather, the authors of D6 studied in theoretical considerations the interference effects between different components of a sample fed to conventional, (multi-component) chromatography. Thus, D6 addresses the problem of preparative and large-scale chromatography which often involves high feed concentrations and large sample volumes (see the introduction section, 1st paragraph on p. 1156 of D6). This was already discussed above under item 3.2 as a possible side-effect of conventional elusion chromatography, but basically contrasts to the principle of displacement chromatography involving the use of a displacer. Therefore, it is believed that reference D6 would not be considered at all by a non-biased person skilled in the

art, i.e. without following an inappropriate hindsight approach, for assessing inventive step.

4.5.2 It is questionable whether document D5 would have been considered at all in view of the problem posed. This document relates to displacement chromatography, but apparently deals with the purification of biopolymers (see title). Also in the description of the practical advantages on p. 20, protein purification is of major concern and, moreover, benefits are reported mainly for analytical separations. Product concentration (not product purity!) is mentioned as a second practical advantage. The review of D5 finalizes with a reference to serious concerns in the art of displacement chromatography to become a practical approach for the separation of biopolymers. It is referred to a "mystery" and "an aura of complexity that can only be overcome with difficulty" being widely associated with displacement chromatography.

Therefore, it is submitted that neither document D5 provides any suggestion to seriously contemplate displacement chromatography for the high-scale preparative purification of HMG-CoA reductase inhibitors.

4.5.3 Documents D3 and D4 relate more specifically to practical applications of displacement chromatography .

4.5.3.1 However, document D4 addresses the specific problem which may occur in displacement chromatography in that an unknown component of the mixture subjected to chromatography may interfere with the application of a suitable displacement agent (see col. 1, l. 25-48). This specific problem appears to be related to the separation and isolation of protein or amino acid or corresponding biochemical material being known in a mixture of a bio-

chemical sample such as body fluid, fermentation broth or cell lysate (col. 5, l. 65 to col. 6, l. 10 of D4). However, without giving any suggestion for solving the problem which is peculiar for the purification of HMG-CoA reductase inhibitors (see items 4.1 to 4.4 above), the teaching of D4 proposes a non-economical and complicated separation process involving the use of two displacement separation columns and a careful control thereof (see claims, Figs 1 to 4 and related description). Accordingly, the skilled person faced with the problem here is not motivated by D4 to apply displacement chromatography for purifying HMG-CoA reductase inhibitors, especially for realizing purity levels which are required for medical applications and which have already been achieved by purification schemes specifically directed to HMG-CoA reductase inhibitors.

4.5.3.2 Document D3 relates to a method for purifying low-molecular weight compounds of a specific peptide or pseudo-peptide structure. The method is based on displacement chromatography, but the method features are specifically designed to purify tuftsin or analogues thereof (see p. 2, l. 14-31, example, claims 1 and 2 of D4). Accordingly, the purification scheme is designed on the basis of ion exchange displacement chromatography in view of the peptide/ pseudo-peptide compound to be purified, which have 1, 2 or 3 more protonable basic functions than the number of acid functions. This is a basically different concept which does not appear to be applicable to HMG-CoA reductase inhibitors. The characteristic structural moiety of HMG-CoA reductase inhibitors is the presence of a basic β,δ-dihydroxyheptanoic acid structure (either in the free acid or in the lactone form) having varying terminal organic residues which conventionally do not have protonable basic functions with a number greater than that of the acid

functions. That is, it should be acknowledged that the description of a practical application of displacement chromatography in line with D3 is not suitable for the purification of HMG-CoA reductase inhibitors.

Furthermore, though some general advantages of displacement chromatography over elusion chromatography are mentioned in D3 (p. 2, l. 48-52), it is to be emphasized that the purity dimension reported in the Example for a specific tuftsin compound ("exceeding 95 %") is significantly below the ultimate high purity required for providing an effective HMG-CoA reductase inhibitor drug as mentioned above. As such an extremely high purity is the key requirement, the skilled person would not receive an incentive from D3 or any one of D4 to D6 to apply displacement chromatography for purifying HMG-CoA reductase inhibitors.

4.6 It should be further acknowledged that, surprisingly, the ultimate purity level was realized by the present invention on at least as high a level as in the HPLC-based process of D1 (which chromatographic method was conventionally recognized to rank among the best chromatographic techniques), and even without a crystallization step being still required according to D1.

4.7 For a proper assessment of inventive step, the following observations should also be taken into account.

The displacement chromatography is known since 1943 (A. Tiselius, Akr. Chemi. Mineral. Geol., 16(A) No. 18 (1943), p. 1 ff. as cited in the present application), and the first HMG-CoA reductase compounds (compactin) were known since 1975 (US-A-3 983 140). Thus, displacement chromatography and HMG-CoA reductase inhibitors have co-existed for more than 25 years. During these 25 years, neither any

guesswork nor any practical attempt was described to purify HMG-CoA reductase inhibitors by means of displacement chromatography. This may be attributable to a general understanding that, even if the purification may become more convenient, the high quality standard of the product can only be reached by the conventional methods applied in the filed of HMG-CoA reductase inhibitors, especially HPLC chromatography, elusion flash and crystallization and re-crystallization processes. In advance, it cannot be fore-seen whether displacement chromatography efficiently works, let alone for providing compounds of such a purity level which is achievable by chromatography based, for example, on HPLC.

4.8 For the above reasons, it is submitted that the use of displacement chromatography involving the use of a dis-placer specifically for purifying HMG-CoA reductase inhibi-tors, as defined by the present claim 1, is not rendered obvious by the referenced prior art.

4.9 In the written opinion, the role of more specific subject-matter was briefly mentioned (i.e. compounds of specific structure to be purified, defined displacers to be used).

However, since the displacement chromatography was applied according to the present invention to both typical types of HMG-CoA reductase inhibitors (i.e. various statins in the salt form and statins in the lactone form), and since numerous different types of displacer compounds were sur-prisingly effective for realizing the required ultimate purity level (see the description of the examples showing representative examples of statins and displacer com-pounds), the inventive concept is well supported within the whole ambit of claim 1.

4.10 Hence, due to the lack of corresponding disclosures or suggestions in the prior art which could provide a hint to the present invention, the subject-matter of the present claim 1 clearly involves an inventive step.

5. Further Observations

Under item 5 of the Communication, it is considered that the definition of the stationary phase as C-18 or C-8 (claim 17) seems unclear. However, it is believed that these indications are quite usual for reversed phase chromatography column materials. Since claim 17 refers to claim 15 which indicates the reverse phase chromatography system, it is submitted that the meaning of C-18 and C-8 is properly understood by the skilled person from the context.

6. Thus, since the claims on file meet the requirements of the PCT in view of the above observations, it is respectfully requested that positive statements be communicated in the international preliminary examination report.

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Encls.:
New specification pages 4 and 16-19

341; J. Chromatogr., 454 (1988) 1 (theoretic optimisation)); A. Felinger et al., J. Chromatogr., 609 (1992) 35 (theoretic optimisation), all papers being introduced herein by way of reference) similar columns were used; the mobile phase was methanol in the phosphate buffer, the displacer was 2-(2-t-butoxyethoxy)ethanol (BEE) in acetonitrile and sodium acetate. Different peptides, proteins and cephalosporin C antibiotic were used as the samples.

US Pat. No. 5,043,4~~92~~23 (27.08.1991) and EP 416.416, respectively, describe the method for purifying certain low molecular (below 1000 daltons) peptides (in particular, tuftsin and synthetic derivatives thereof) with displacement ion-exchange chromatography where the stationary phase used is cationic-exchange resin, the transporter solvent is water or dilute solutions of a variety of strong acids, and the displacer used is triethylenetetraammonuim salt in different concentrations. In US patent application 08/875,422, yet unpublished, the use of displacement chromatography for the isolation and purification of vancomycin is described.

Technical Solution

It is sometimes difficult to obtain the active substance of high purity in a large scale as many technologies applicable to a laboratory scale are not sufficiently economical in large scale production operations to justify use thereof or do not meet the environmental criteria. The above facts compel the industry to search for new technologies that will provide both the high-quality product and the economically and ecologically acceptable production.

The present invention has solved the drawbacks of the processes known from the older patents and other literature as it enables to obtain the pure HMG-CoA

Claims

1. A process for obtaining HMG-CoA reductase inhibitors, characterised in that one of the steps in the process of the purification of crude HMG-CoA reductase inhibitors includes displacement chromatography which involves the use of a displacer for displacing the HMG-CoA reductase inhibitor.

2. A process according to claim 1, characterised in that the HMG-CoA reductase inhibitor is selected from the group consisting of mevastatin, pravastatin, lovastatin, simvastatin, fluvastatin and atorvastatin.

3. A process according to claim 1 or 2, characterised in that the HMG-CoA reductase inhibitor is in the lactone form or in the form of the acid or the salt thereof.

4. A process according any one of claims 1 to 3, characterised in that the displacement chromatography includes the following steps:
a) conditioning a chromatography column with a mobile phase,
b) feeding HMG-CoA reductase inhibitor dissolved in the mobile phase,
c) introducing the displacer for displacing the HMG-CoA reductase inhibitor from the column, and
d) obtaining the purified HMG-CoA reductase inhibitor.

5. A process according to claim 4, characterised in that the purified HMG-CoA reductase inhibitor is obtained by
d1) collecting the fractions, and
d2) analyzing the fractions with analytical HPLC and pooling the fractions depending on the quality of purity.

6. A process according to claim 4 or 5, characterised in that the displacement chromatography further includes the subsequent step of:

e) regenerating the chromatography column by washing the column with alcohol/water mixture to elute the displacer.

7. A process according to claim 4, characterised in that the mobile phase is selected from the group of solvents consisting of water, acetonitrile/water solutions or aqueous solutions of lower alcohols, as well as bufferred dilute solutions of organic, halogenated organic or inorganic acids with alkaline metal cations, with ammonia or with amines.

8. A process according to claim 7, characterised in that the mobile phase is any one of water, an acetonitrile/water solution or an aqueous solution of lower alcohols.

9. A process according to claim 4, characterised in that the pH of the mobile phase used is between 4.5 and 10.5.

10. A process according to claim 9, characterised in that the pH of the mobile phase used is between 6.5 and 8.

11. A process according to claim 10, characterised in that the pH of the mobile phase used is 7.

12. A process according to claim 4, characterised in that the flow rate of the mobile phase through the chromatographic column is between 1.5 and 30 ml/(min cm^2).

13. A process according to claim 4, characterised in that the flow rate of the mobile phase/displacer mixture through the chromatographic column is between 3 and 15 ml/(min cm^2).

14. A process according to claim 6, characterised in that the stationary phase is regenerated with 20 to 100% aqueous solution of lower alcohols after completed chromatography.

15. A process according to claim 4, characterised in that the stationary phase is a reverse phase.

16. A process according to claim 15, characterised in that the stationary phase is a natural reverse phase such as silica gel with alkyl chains of different lengths.

17. A process according to claim 15, characterised in that the stationary phase is either C-18 or C-8.

18. A process according to claim 15, characterised in that the stationary phase is a synthetic cross-linked polymer matrix.

19. A process according to claim 18, characterised in that the cross-linked polymer matrix is a copolymer of styrene and divinylbenzene.

20. A process according to claim 4, characterised in that the particle size of the stationary phase is between 3 and 20 µm.

21. A process according to claim 20, characterised in that the particle size of the stationary phase is between 7 and 15 µm.

22. A process according to claim 4, characterised in that the displacer is selected from the group consisting of long chain alcohols, long chain carboxylic acids, long chain alkyl ammonium salts, aromatic dicarboxylic acid

esters, oxo- and dioxo-alcohols, polyalkylene polyglycol ethers and polyaryl or polyalkylene polyaryl ethers.

23. A process according to claim 4, characterised in that the concentration of the displacer in the mobile phase is between 1 and 35%.

24. A process according to claim 23, characterised in that the concentration of the displacer in the mobile phase is between 2 and 20%.

25. The use of a process according to any one of claims 1 to 24 for producing a HMG-CoA reductase inhibitor with a HPLC purity exceeding 99.7%.

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WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C07D 309/30, 405/06, B01D 15/08

A1

(11) International Publication Number: WO 00/17182

(43) International Publication Date: 30 March 2000 (30.03.00)

(21) International Application Number: PCT/IB99/01553

(22) International Filing Date: 17 September 1999 (17.09.99)

(30) Priority Data:
P-9800241 18 September 1998 (18.09.98) SI

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: PROCESS FOR OBTAINING HMG-CoA REDUCTASE INHIBITORS OF HIGH PURITY

(57) Abstract

Lovastatin, pravastatin, simvastatin, mevastatin, atorvastatin, and derivatives and analogs thereof are known as HMG-CoA reductase inhibitors and are used as antihypercholesterolemic agents. The majority of them are produced by fermentation using microorganisms of different species identified as species belonging to *Aspergillus*, *Monascus*, *Nocardia*, *Amycolatopsis*, *Mucor* or *Penicillium genus*, some are obtained by treating the fermentation products using the method of chemical synthesis or they are the products of total chemical synthesis. The purity of the active ingredient is an important factor for manufacturing the safe and effective pharmaceutical, especially if the pharmaceutical product must be taken on a longer term basis in the treatment or prevention of high plasma cholesterol. The accumulation of the impurities from the pharmaceuticals of lower purity may cause many side effects during the medical treatment. The present invention relates to a new industrial process for the isolation of HMG-CoA reductase inhibitors using so-called displacement chromatography. Use of the invention enables to obtain HMG-CoA reductase inhibitors of high purity, with high yields, lower production costs and suitable ecological balance.

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Title of the invention

Process for obtaining HMG-CoA reductase inhibitors of high purity

Technical Field

Lovastatin, pravastatin, simvastatin, mevastatin, atorvastatin and derivatives and analogs thereof are known as HMG-CoA reductase inhibitors and are used as antihypercholesterolemic agents. The majority of them are produced by fermentation using microorganisms of different species identified as species belonging to *Aspergillus, Monascus, Nocardia, Amycolatopsis, Mucor* or *Penicillium* genus, some are obtained by treating the fermentation products using the method of chemical synthesis or they are the products of total chemical synthesis.

The purity of the active ingredient is an important factor for manufacturing the safe and effective pharmaceutical, especially if the pharmaceutical product must be taken on a longer term basis in the treatment or prevention of high plasma cholesterol. The accumulation of the impurities from the pharmaceuticals of lower purity may cause many side effects during the medical treatment.

The present invention relates to a new industrial process for the isolation of HMG-CoA reductase inhibitors using so-called displacement chromatography. Use of the invention enables to obtain HMG-CoA reductase inhibitors of high purity, with high yields, lower production costs and suitable ecological balance.

Prior Art

The processes for the isolation and purification of antihypercholesterolemic agents disclosed in the earlier patents include a variety of combinations of extraction, chromatography, lactonisation and crystallisation methods. The purity of the final product obtained by these procedures comply with the USP standards but the yields of the desired product are relatively low. In addition, they require both large amounts of organic solvents and the large equipment suited for these quantities.

The isolation process disclosed in WO 92/16276 provides the solution for obtaining HMG-CoA reductase inhibitors of purity greater than 99.5% with the use of industrial HPLC (high performance liquid chromatography) equipment. According to WO 92/16276 the crude HMG-CoA reductase inhibitor, with a purity of ≥ 85%, is dissolved in an organic solvent or in a solution of organic solvent and water. The mixture is then buffered to a pH between 2 and 9 and placed on the HPLC column. After the HMG-CoA reductase inhibitor peak of interest is collected, a portion of solvent is removed and the water is added or alternatively two-thirds of the solvent mixture are removed and the HMG-CoA reductase inhibitor is crystallised. At the end, the purity of the product obtained by this process is at least 99.5% with the yield of around 90%.

The method disclosed in WO 92/16276 enables obtaining of HMG-CoA reductase inhibitors of high purity, with relatively high yields, the disadvantage of the method over the conventional chromatography columns are relatively small quantities of the substance loaded per HPLC column. Small samples to be fed into the column are also related with increased number of repetitions of the isolation operations in order to obtain sufficient quantities of the desired substance, and consequently

large amount of the solvents used resulting in higher production costs.

Displacement chromatography method, the basis of the present invention, does not substantially differ from previously used chromatography methods.

Displacement chromatography is based on competition of the components of the sample fed into the column for active sites on the stationary phase. Individual components of the sample displace one another like a train, the displacer, having the very high affinity for the stationary phase and travelling behind the fed sample along the column, drives the separation of the sample components into one-compartment zones which move at the same velocity as the displacer. Concentrating of individual components is carried out simultaneously with the purification.

The principle of displacement chromatography method is relatively old as it has been known since 1943 but it was introduced into practice as late as 1981 because of the lack of efficient columns (Cs. Horvath et al., J. Chromatogr., 215 (1981) 295; J. Chromatogr., 330 (1985) 1; J. Chromatogr., 440 (1988) 157). These papers, introduced herein by way of reference, describe the analytic and preparative separation and purification of biologically active peptides and polymyxin antibiotics (polypeptides) using reversed-phase high performance liquid chromatography columns in the displacement mode. For polymyxins octadecyl silica gel columns 250 x 4.6 mm, particle size 5μm, 10% acetonitrile in water as the mobile phase and different tetraalkylammonium halogenides as the displacer were used.

In recent investigations in the field of displacement chromatography (S.M. Cramer et al., Enzyme Microb. Technol., 11 (1989) 74; Prep. Chromatogr., 1 (1988) 29; J. Chromatogr., 394 (1987) 305; J. Chromatogr., 439 (1988)

341; J. Chromatogr., 454 (1988) 1 (theoretic optimisation)); A. Felinger et al., J. Chromatogr., 609 (1992) 35 (theoretic optimisation), all papers being introduced herein by way of reference) similar columns were used; the mobile phase was methanol in the phosphate buffer, the displacer was 2-(2-t-butoxyethoxy)ethanol (BEE) in acetonitrile and sodium acetate. Different peptides, proteins and cephalosporin C antibiotic were used as the samples.

US Pat. No. 5,043,432 (27.08.1991) and EP 416.416, respectively, describe the method for purifying certain low molecular (below 1000 daltons) peptides (in particular, tuftsin and synthetic derivatives thereof) with displacement ion-exchange chromatography where the stationary phase used is cationic-exchange resin, the transporter solvent is water or dilute solutions of a variety of strong acids, and the displacer used is triethylenetetraammonuim salt in different concentrations. In US patent application 08/875,422, yet unpublished, the use of displacement chromatography for the isolation and purification of vancomycin is described.

Technical Solution

It is sometimes difficult to obtain the active substance of high purity in a large scale as many technologies applicable to a laboratory scale are not sufficiently economical in large scale production operations to justify use thereof or do not meet the environmental criteria. The above facts compel the industry to search for new technologies that will provide both the high-quality product and the economically and ecologically acceptable production.

The present invention has solved the drawbacks of the processes known from the older patents and other literature as it enables to obtain the pure HMG-CoA

reductase inhibitors and, additionally, the purifying process *per se* is not time-consuming providing high yields, using small amounts of solvents. The process is nature friendly; in addition, it is not demanding in terms of space and energy thus enabling an economical large scale production.

Description of the invention

The present invention provides a process for the purification of HMG-CoA reductase inhibitors employing displacement chromatography. That is, at least one of the steps in the process of the purification of crude HMG-CoA reductase inhibitor includes displacement chromatography.

The HMG-CoA reductase inhibitor to be purified is, for example, selected from the group consisting of mevastatin, pravastatin, lovastatin, simvastatin, fluvastatin and atorvastatin. The selected inhibitor may be in the lactone form or in the form of the acid or the salt thereof for being purified by means of displacement chromatography.

The displacement chromatography being characteristic for the process of the present invention preferably includes the following steps:

a) conditioning a chromatography column with an appropriate mobile phase,

b) feeding the crude HMG-CoA reductase inhibitor dissolved in the mobile phase,

c) introducing the displacer for displacing the HMG-CoA reductase inhibitor from the column, and

d) obtaining the purified HMG-CoA reductase inhibitor.

The purified HMG-CoA reductase inhibitor is preferably obtained by

d1) collecting the fractions and

d2) analyzing the fractions with analytical HPLC and pooling the fractions depending on the quality of purity.

After the purified HMG-CoA reductase inhibitor has been obtained, the chromatography column may be regenerated by washing of the column with alcohol/water mixture to elute the displacer.

HMG-CoA reductase inhibitors obtained in the herein-described manner are then isolated from the mobile phase according to the methods already known from the state of prior art, for example by lyophilisation or, prefarably, by crystallization to obtain the lactone form, the acid form or the salt form (preferably alkaline or earth alkaline salts) thereof.

The fractions containing a considerable percentage of HMG-CoA reductase inhibitors, in addition to impurities, may be re-subjected to the process resulting in the total yield exceeding 95%.

The stationary phase used is a reverse phase where natural (silica gel with alkyl chains of a different length) or synthetic (C-18 or C-8 organic) stationary phases are suitable. Preferably, a synthetic cross-linked polymer matrix of styrene and divinylbenzene is used. The particle size of the stationary phase is suitably from 3 to 20 µm, preferably between 7 and 15 µm.

The mobile phase used is preferably selected from water, acetonitrile/water solution and aqueous solutions of lower (preferably C_1-C_4) alcohols, buffered dilute solutions of organic, halogenated organic or inorganic acids, e.g. formic, acetic, propionic, hydrochloric, boric, phosphoric, carbonic or suphuric acids with cations of alkaline metals, with ammonia or with amines. Water and aqueous solutions with acetonitrile and especially with methanol or ethanol are particularly preferred, and the content of the organic solvent in the aqueous solutions preferably is 80% or below, more preferably 45% or below

and particularly 30% or below. Since toxic methanol in the mobile phase may be replaced by less toxic ethanol, or may be at least partially replaced by water with good results, removal of waste solvents is simpler, therefore, the present invention is a marked improvement compared to the state of prior art judging from the ecological aspect.

The pH of the mobile phase used is preferably between 4.5 and 10.5, more preferably between 6.5 and 8, and particularly around 7. The flow rate of the mobile phase through the column is suitably adjusted to lie between 1.5 and 30 ml/(min cm^2), preferably between 3 and 15 ml/(min cm^2). At the time when the displacer is introduced into the chromatography column by being mixed with the mobile phase, the flow rate is preferably adjusted to lie between 1.5 and 15 ml/(min cm^2) and particularly between 3 and 10 ml/(min cm^2), because higher flow rates cause the dilution of the samples to be collected, and also the separation becomes worse.

The displacer suitably is a compound having an amphiphilic structure, such as surfactants, detergents and the like. Examples of the displacer are long chain alcohols, long chain carboxylic acids, long chain alkyl ammonium salts, aromatic dicarboxylic acid esters, oxo- and dioxo-alcohols, polyalkylene polyglycol ethers such as diethylene glycol mono- (or di-)alkylethers, polyaryl or polyalkylene polyaryl ethers such as Triton® X-100, etc. The aforementioned "long chain" means an alkyl chain having at least a C_4-chain, preferably at least a C_{10}-chain and more preferably at least a C_{14}-chain or longer.

The concentration of the displacer in the mobile phase is suitably adjusted to be from 1 to 35%, preferably from 2 to 20% and particularly from 7 to 14%.

In the preferred embodiment of controlling the quality of purity in the individual fractions eluted from the chromatography column, an analytical HPLC method directed

to the HMG-CoA reductase inhibitors to be analyzed may be carried out as described in the following.

The sample to be analysed is diluted 100 times with the mobile phase containing 20 mM aqueous NH_4HCO_3 solution with acetonitrile (the proportion of acetonitrile is adjusted such that the retention factor of the analyte is between 5 and 10). 10 µl of this sample is placed on Hypersil ODS column (Hypersil, the United Kingdom, particle size 3µm, column size 50 × 4.6 mm) for high performance liquid chromatography. The column is washed with the mobile phase at the flow rate of 2 ml/min. Absorbance is measured at 235 nm. HPLC purity of the sample is calculated from the ratio between the areas of individual peaks in the chromatogram.

After completed chromatography the stationary phase is preferably regenerated, for example using the mobile phase with 20 to 100% aqueous solution of lower alcohol.

The invention is illustrated but in no way limited by the following examples.

EXAMPLES

Example 1

Crude sodium salt of pravastatin (1.0 g, HPLC purity 88%, assay 85%) was dissolved in 10 ml of the mobile phase A (distilled water), pH was adjusted to 7 with 0.2M aqueous NaOH solution and filtered. The column was equilibrated with mobile phase A. The sample obtained in the above-described manner was fed onto the Grom-Sil 120-ODS HE column (Grom Analytic + HPLC GmbH, Germany), particle size 11 µm, column size 250 × 10 mm. The column was washed with the mobile phase B containing 7% of diethyleneglycol monobutylether in mobile phase A at the flow rate of 4.5 ml/min. Absorbance was measured at 260 nm, and the 0.5 ml fractions were collected with an initial increase in the

absorbance. When the signal decreased the column was washed with 25 ml of 70% methanol. The obtained fractions were analyzed by the herein above-described HPLC analytical method. The fractions with a purity ≥ 99.5% were pooled. In the pooled fractions (7 ml) the HPLC purity was 99.8%.

Example 2

Crude sodium salt of pravastatin (0.4 g, HPLC purity 88%, assay 85%) was dissolved in 5 ml of the mobile phase A (distilled water), pH was adjusted to 7 with 0.2M aqueous NaOH solution and filtered. The column was equilibrated with mobile phase A. The sample obtained in the above-described manner was fed onto the Kromasil 100 C-18 column (EKA Chemicals AB, Sweden), particle size 10 μm, column size 200 × 10 mm. The column was washed with the mobile phase B containing 7% of Triton X-100 in mobile phase A at the flow rate of 1 ml/min. Absorbance was measured at 260 nm, and the 0.5 ml fractions were collected with an initial increase in the absorbance. The obtained fractions were analysed by the above described HPLC analytical method. The fractions with a purity ≥ 99.5% were pooled. In the pooled fractions (3 ml) the HPLC purity was 99.7%.

Example 3

0.6 g of the crude sodium salt of pravastatin was dissolved in 5 ml of distilled water. The protocol described in Example 1 was used with the exception of the mobile phase used (30% aqueous methanol solution) and the pooled fractions with a HPLC purity of 99.8% were obtained.

Example 4

The method described in Example 3 was repeated wherein the concentration of the displacer in the mobile phase was 14%. In the fractions pooled, according to the criterion described in Example 1, HPLC purity was 99.8%.

Example 5

Pravastatin lacton (0.4g, HPLC purity 85%) was dissolved in 33 ml of the mobil phase A containing 45% methanol. The column was equilibrated with mobile phase A. The sample obtained in the above-described manner was fed onto the Grom-Sil 120-ODS HE column (Grom Analytic + HPLC GmbH, Germany), particle size 11 μm, column size 250 × 10 mm. The column was washed with the mobile phase B containing 2% of diethyleneglycoldibutylether in mobile phase A at the flow rate of 4.5 ml/min. Absorbance was measured at 260 nm, and the 1ml fractions were collected with an initial increase in the absorbance. When the signal decreased the column was washed with 25 ml of 70% methanol.

The fractions with a purity ≥ 99.5% were pooled. In the pooled fractions the HPLC purity was 99.7%.

Example 6

Pravastatin lacton (0.3g, HPLC purity 85%) was dissolved in 80 ml of the mobil phase A containing 30% methanol. The column was equilibrated with mobile phase A. The sample obtained in the above-described manner was fed onto the Licrosphere RP 18 column, particle size 12 μm, column size 200 × 10 mm. The column was washed with the mobile phase B containing 5% of diethyleneglycolmono-n-hexylether in mobile phase A at the flow rate of 4.5 ml/min. Absorbance

was measured at 235 nm, and the 1ml fractions were collected with an initial increase in the absorbance. When the signal decreased the column was washed with 25 ml of 90% methanol. The obtained fractions were analysed by the above described HPLC analytical method.

The fractions with a purity ≥ 99.5% were pooled. In the pooled fractions the HPLC purity was 99.8%.

Example 7

Pravastatin lacton (0.3g, HPLC purity 85%) was dissolved in 25 ml of the mobil phase A containing 35% acetonitrile. The column was equilibrated with mobile phase A. The sample obtained in the above-described manner was fed onto the Licrosphere RP 18 column, particle size 12 µm, column size 200 × 10 mm. The column was washed with the mobile phase B containing 1% of diethyleneglycoldibutylether in mobile phase A at the flow rate of 4.5 ml/min. Absorbance was measured at 235 nm, and the 1ml fractions were collected with an initial increase in the absorbance. When the signal decreased the column was washed with 25 ml of 90% methanol. The obtained fractions were analysed by the above described HPLC analytical method.

The fractions with a purity ≥ 99.5% were pooled. In the pooled fractions the HPLC purity was 99.8%.

Example 8

The method described in Example 7 was repeated wherein the mobile phase B was 0.85% diethylphthalat in the mobile phase A.

The fractions with a purity ≥ 99.5% were pooled. In the pooled fractions the HPLC purity was 99.8%.

Example 9

Simvastatin lacton (0.42g, HPLC purity 87%) was dissolved in 6 ml of the 66% acetonitrile and hydrolysed with 1.2mmol of sodium hydroxide. Acetonitrile was removed and pH was adjusted to 7 with diluted H_3PO_4. The column was equilibrated with mobile phase A containing 14% of methanol. The sample obtained in the above-described manner was fed onto the Grom-Sil 120-ODS HE column (Grom Analytic + HPLC GmbH, Germany), particle size 11 μm, column size 250 × 10 mm. The column was washed with the mobile phase B containing 6.7% of diethyleneglycolmono-n-hexylether in mobile phase A at the flow rate of 4.5 ml/min. Absorbance was measured at 260 nm, and the 0.5ml fractions were collected with an initial increase in the absorbance. When the signal decreased the column was washed with 25 ml of methanol.

The fractions with a purity ≥ 99.5% were pooled. In the pooled fractions the HPLC purity was 99.8%.

Example 10

Simvastatin lacton (0.5g, HPLC purity 87 %) was dissolved in 20 ml of the mobile phase containing 70% of methanol. The column was equilibrated with mobile phase A. The sample obtained in the above-described manner was fed onto the Grom-Sil 120-ODS HE column (Grom Analytic + HPLC GmbH, Germany), particle size 11 μm, column size 250 × 10 mm. The column was washed with the mobile phase B containing 3% of decanoic acid in mobile phase A at the flow rate of 4.5 ml/min. Absorbance was measured at 260 nm, and the 0.75 ml fractions were collected with an initial increase in the absorbance. When the signal decreased the column was washed with 25 ml of methanol. The obtained fractions were analyzed by the herein above described method. The fractions with a purity ≥ 99.5% were pooled. In the pooled fractions the HPLC purity was 99.7%.

Example 11

Simvastatin lacton (0.5 g, HPLC purity 87%) was dissolved in 20 ml of the mobile phase containing of 60% acetonitrile. The column was equilibrated with mobile phase A . The sample obtained in the above-described manner was fed onto the Grom-Sil 120-ODS HE column (Grom Analytic + HPLC GmbH, Germany), particle size 11 μm, column size 250 × 10 mm. The column was washed with the mobile phase B containing 2% of tetrakis(decyl)amonium bromide in mobile phase A at the flow rate of 4.5 ml/min. Absorbance was measured at 260 nm, and the 1ml fractions were collected with an initial increase in the absorbance. When the signal decreased the column was washed with 25 ml of methanol.

The fractions with a purity ≥ 99.5% were pooled. In the pooled fractions the HPLC purity was 99.8%.

Example 12

Lovastatin lacton (0.5g, HPLC purity 87%) was dissolved in 60 ml of the 75% methanol. The column was equilibrated with mobile phase A containing 70% of methanol. The sample obtained in the above-described manner was fed onto the Grom-Sil 120-ODS HE column (Grom Analytic + HPLC GmbH, Germany), particle size 11 μm, column size 250 × 10 mm. The column was washed with the mobile phase B containing 70% of methanol and 4.5% of decanoic acid in mobile phase A at the flow rate of 6 ml/min. Absorbance was measured at 260 nm, and the 1ml fractions were collected with an initial increase in the absorbance. When the signal decreased the column was washed with 25 ml of methanol.

The obtained fractions were analysed by the above described HPLC analytical method.

The fractions with a purity ≥ 99.5% were pooled. In the pooled fractions the HPLC purity was 99.9%.

Example 13

Lovastatin lacton (0.42g, HPLC purity 87 %) was dissolved in 8 ml of the 50% acetonitrile and hydrolysed with 1.5 mmol of sodium hydroxide. Acetonitrile was removed and pH was adjusted to 7 with diluted H_3PO_4. The column was equilibrated with mobile phase A containing 14% of methanol. The sample obtained in the above-described manner was fed onto the Grom-Sil 120-ODS HE column (Grom Analytic + HPLC GmbH, Germany), particle size 11 µm, column size 250 × 10 mm. The column was washed with the mobile phase B containing 6.7% of diethyleneglycolmono-n-hexylether in mobile phase A at the flow rate of 1 ml/min. Absorbance was measured at 260 nm, and the 0.25 ml fractions were collected with an initial increase in the absorbance. When the signal decreased the column was washed with 25 ml of methanol.

The obtained fractions were analysed by the method described in example 9. The fractions with a purity ≥ 99.5% were pooled. In the pooled fractions the HPLC purity was 99.8%.

Example 14

Mevastatin lacton (0.5g, HPLC purity 85%) was dissolved in 150 ml of the mobile phase A containing 70% of methanol. The column was equilibrated with mobile phase A. The sample obtained in the above-described manner was fed onto the Grom-Sil 120-ODS HE column (Grom Analytic + HPLC GmbH, Germany), particle size 11 µm, column size 250 × 10 mm. The column was washed with the mobile phase B containing 4.5% of decanoic acid in mobile phase A at the flow rate of 6 ml/min. Absorbance was measured at 260 nm, and the

1 ml fractions were collected with an initial increase in the absorbance. When the signal decreased the column was washed with 25 ml of methanol.

The obtained fractions were analysed by the above described HPLC analytical method.

The fractions with a purity ≥ 99.5% were pooled. In the pooled fractions the HPLC purity was 99.8%.

Claims

1. A process for obtaining HMG-CoA reductase inhibitors, characterised in that one of the steps in the process of the purification of crude HMG-CoA reductase inhibitors includes displacement chromatography.

2. A process according to claim 1, characterised in that the HMG-CoA reductase inhibitor is selected from the group consisting of mevastatin, pravastatin, lovastatin, simvastatin, fluvastatin and atorvastatin.

3. A process according to claim 1 or 2, characterised in that the HMG-CoA reductase inhibitor is in the lactone form or in the form of the acid or the salt thereof.

4. A process according any one of claims 1 to 3, characterised in that the displacement chromatography includes the following steps:

a) conditioning a chromatography column with a mobile phase,

b) feeding HMG-CoA reductase inhibitor dissolved in the mobile phase,

c) introducing the displacer for displacing the HMG-CoA reductase inhibitor from the column, and

d) obtaining the purified HMG-CoA reductase inhibitor.

5. A process according to claim 4, characterised in that the purified HMG-CoA reductase inhibitor is obtained by

d1) collecting the fractions, and

d2) analyzing the fractions with analytical HPLC and pooling the fractions depending on the quality of purity.

6. A process according to claim 4 or 5, characterised in that the displacement chromatography further includes the subsequent step of:

e) regenerating the chromatography column by washing the column with alcohol/water mixture to elute the displacer.

7. A process according to claim 4, characterised in that the mobile phase is selected from the group of solvents consisting of water, acetonitrile/water solutions or aqueous solutions of lower alcohols, as well as bufferred dilute solutions of organic, halogenated organic or inorganic acids with alkaline metal cations, with ammonia or with amines.

8. A process according to claim 7, characterised in that the mobile phase is any one of water, an acetonitrile/water solution or an aqueous solution of lower alcohols.

9. A process according to claim 4, characterised in that the pH of the mobile phase used is between 4.5 and 10.5.

10. A process according to claim 9, characterised in that the pH of the mobile phase used is between 6.5 and 8.

11. A process according to claim 10, characterised in that the pH of the mobile phase used is 7.

12. A process according to claim 4, characterised in that the flow rate of the mobile phase through the chromatographic column is between 1.5 and 30 ml/(min cm^2).

13. A process according to claim 4, characterised in that the flow rate of the mobile phase/displacer mixture through the chromatographic column is between 3 and 15 ml/(min cm^2).

14. A process according to claim 6, characterised in that the stationary phase is regenerated with 20 to 100% aqueous solution of lower alcohols after completed chromatography.

15. A process according to claim 4, characterised in that the stationary phase is a reverse phase.

16. A process according to claim 15, characterised in that the stationary phase is a natural reverse phase such as silica gel with alkyl chains of different lengths.

17. A process according to claim 15, characterised in that the stationary phase is either C-18 or C-8.

18. A process according to claim 15, characterised in that the stationary phase is a synthetic cross-linked polymer matrix.

19. A process according to claim 18, characterised in that the cross-linked polymer matrix is a copolymer of styrene and divinylbenzene.

20. A process according to claim 4, characterised in that the particle size of the stationary phase is between 3 and 20 µm.

21. A process according to claim 20, characterised in that the particle size of the stationary phase is between 7 and 15 µm.

22. A process according to claim 4, characterised in that the displacer is selected from the group consisting of long chain alcohols, long chain carboxylic acids, long chain alkyl ammonium salts, aromatic dicarboxylic acid

esters, oxo- and dioxo-alcohols, polyalkylene polyglycol ethers and polyaryl or polyalkylene polyaryl ethers.

23. A process according to claim 4, characterised in that the concentration of the displacer in the mobile phase is between 1 and 35%.

24. A process according to claim 23, characterised in that the concentration of the displacer in the mobile phase is between 2 and 20%.

25. The use of a process according to any one of claims 1 to 24 for producing a HMG-CoA reductase inhibitor with a HPLC purity exceeding 99.7%.

26. HMG-CoA reductase inhibitor with a HPLC purity exceeding 99.7% obtained by purifying crude HMG-CoA reductase inhibitor by means of a purification process which includes displacement chromatography.

27. The substance according to claim 26, characterized in that the HMG-CoA reductase inhibitor is selected from the group consisting of lovastatin, simvastatin, pravastatin, atorvastatin, mevastatin and fluvastatin.

28. The substance according to claim 27, characterized in that the selected HMG-CoA reductase inhibitor is lovastatin, simvastatin or pravastatin.

29. The substance according to claim 26, characterized in that the selected HMG-CoA reductase inhibitor is in the lactone form or in the form of the acid or the salt thereof.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB 99/01553

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07D309/30 C07D405/06 B01D15/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07D B01D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	the whole document	1-25
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
16 November 1999	23/11/1999
Name and mailing address of the ISA	**Authorized officer**
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Steendijk, M

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International Application No
PCT/IB 99/01553

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International Application No
PCT/IB 99/01553

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Form PCT/ISA/210 (patent family annex) (July 1992)

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
18 January 2001 (18.01.2001)

PCT

(10) International Publication Number
WO 01/04340 A1

(51) International Patent Classification[7]: C12P 17/06, 7/42

(21) International Application Number: PCT/HU00/00066

(22) International Filing Date: 29 June 2000 (29.06.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
P 9902352 12 July 1999 (12.07.1999) HU

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(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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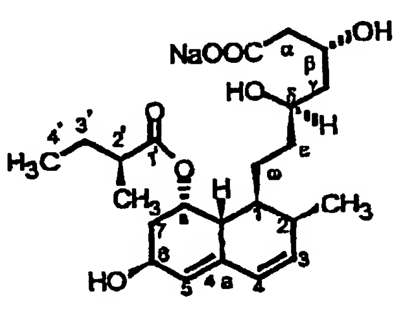

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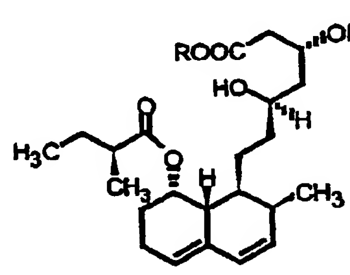


(II)

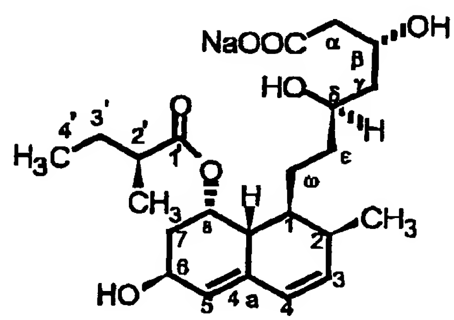
(57) Abstract: The present invention relates to a new microbial process for the preparation of compound of formula (I) from a compound of general formula (II) wherein R stands for an alkali metal or ammonium ion, by the submerged culture of a strain which is able to 6β-hydroxylate the compound of formula (II) in aerobic fermentation and by the separation and purification of the product of formula (I) formed in the course of the bioconversion. The latter comprises the cultivation of a *Micromonospora* strain which is able to 6β-hydroxylate a compound of general formula (II) - wherein R is as defined above - at 25-32 °C on a nutrient medium containing available carbon - and nitrogen sources and mineral salts, thereafter feeding the substrate to be transformed into the developing culture, then hydroxilating the substrate until finishing of the bioconversion, then separating the compound of formula (I) from the culture broth and, if desired, purifying the same.

WO 01/04340 A1

HYDROXYLATION OF COMPACTIN TO PRAVATATIN BY MICROMONOSPORA

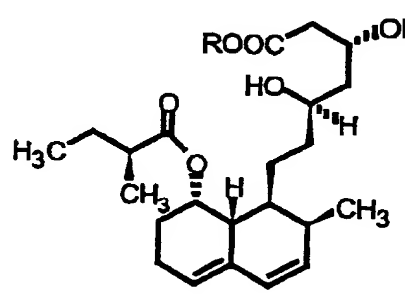
The present invention relates to a new microbial process for the preparation of pravastatin.

More particularly, this invention relates to a microbial process for the preparation of pravastatin of formula (I)



(I)

from a compound of the general formula (II)



(II)

wherein R stands for an alkali metal or ammonium ion, with a microorganism, wherein said microorganism is a prokaryote from genus *Micromonospora*, which is able to hydroxylate a compound of the general formula (II) at the 6β-position.

The hypercholesterolaemia has been recognized as a major risk factor for atherosclerotic disease, specifically for coronary heart disease. During the past two decades 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase EC. 1.1.1.34) as the major rate-limiting enzyme in the cholesterol biosynthesis, has been extensively studied. Mevinolin and related compounds biosynthesised by selected strains of different fungal species were found to be competitive inhibitors of this enzyme [Endo, A. et al., J. Antibiotics **29**, 1346-1348 (1976); Endo, A. et al., FEBS Lett. **72**, 323-326 (1976): Kuo, C.H. et al., J. Org. Chem. **48**, 1991-1998 (1983)].

Pravastatin is also a member of the family of HMG-CoA reductase inhibitors. At first, pravastatin was found as a minor urinary metabolite of compactin in dog (Tanaka, M. et al., unpublished) in the course of metabolic studies of compactin [Arai, M. et al. Sankyo Kenkyusho Nempo, **40**, 1-38 (1988)].

The main characteristic property of pravastatin as the hydroxylated product of compactin is its tissue selectivity. This drug strongly inhibits sterol synthesis in liver and in intestine, but weakly in other organs. It is advantageous that pravastatin possesses lower toxicity than the other HMG-CoA reductase inhibitors.

It has been reported that microbial hydroxylation of compactin can be accomplished in various extent by several strains of species belonging to many different genera of fungi, and by strains of actinomycete species belonging to the genera *Nocardia, Actinomadura* and *Streptomyces,* among others *Streptomyces roseochromogenes* and *Streptomyces carbophilus* (U.S. Patent No. 5,179,013, U.S. Patent No. 4,448,979, U.S. Patent No. 4,346,227, U.S. Patent No. 4,537,859, Japanese Patent No. 58,010,572).

A problem with using fungi for the production of pravastatin from compactin is that these organisms generally do not tolerate higher concentrations of compactin in liquid culture media, presumably due to its antifungal activity [Serizawa, N. et al., J. Antibiotics **36**, 887-891 (1983)]. In *Streptomyces carbophilus* the cytochrome P450 system has been shown to be required for the hydroxylation of compactin to pravastatin [Matsuoka, T. et al., Eur. J. Biochem. **184**, 707-713 (1989)]. Difficulty of genetic improvement of the ability

of hydroxylation with the use of such an enzyme is that it is a complex of proteins rather than a single protein.

Our investigation were focused on finding an actinomycete strain which would produce pravastatin from salts of acidic form of compactin with higher yield and by applying higher substrate concentration in the bioconversion than those known from former patent specifications.

During the screening, covering about 6000 actinomycetes, mostly our own isolates, but also authentic strains from international strain collections, five *Streptomyces* and five *Micromonospora* were selected for further studies, because they proved to be able to hydroxylate the sodium salt of the acidic form of compactin into pravastatin. These ten actinomycete strains, from which eight strains have been taxonomically identified at species level in our laboratory, were the following:

Streptomyces violaceus (according to Kämpfer et al, 1991), strain No. 1/43.
Streptomyces rochei (Berger et al., 1949; Waksman and Lechevalier, 1953), strain No. 1/41.
Streptomyces resistomycificus (Lindenbein, 1952), strain No. 1/44.
Streptomyces sp., strain No. 1/28.
Streptomyces lanatus, (Frommer, 1959), strain No. 1/16.
Micromonospora sp., strain No. IDR-P_3.
Micromonospora purpurea (Luedemann and Brodsky, 1964), strain No. IDR-P_4.
Micromonospora echinospora (Luedemann and Brodsky, 1964), strain No. IDR-P_5.
Micromonospora megalomicea (Weinstein et al, 1969), strain No. IDR-P_6.
Micromonospora rosaria (Horan and Brodsky, 1986), strain No. IDR-P_7.

Since, up to now, there are no data in the literature on the ability of *Micromonospora* to convert salts of the acidic form of compactin into pravastatin, we have thoroughly studied not only this particular enzymatic ability, but also the taxonomic position of these above listed strains of *Micromonospora*.

Taxonomic position of strains IDR-P_3, -P_4, -P_5, -P_6 and –P_7 at generic level

All of these strains produced well developed mycelia, composed of branched hyphae of about 0.4-0.7 μm in diameter. Aerial mycelium is absent or occurs only in traces. Nonmotile spores are borne on sporophores singly. Hyphae of the substrate mycelium are Gram-positive and not acid-fast. Strains Nos. IDR P_3–P_7 are aerobic, chemo-organotrophic and sensitive to pH below 6.0. Walls contain meso-diaminopimelic acid. The above listed diagnostic properties – as key characters – clearly demonstrate, that these monosporic actinomycete strains are typical members of the genus *Micromonospora.*

Taxonomic description of Micromonospora sp., strain No. IDR-P_3

Micromorphological properties: Substrate mycelium is composed of well developed, more curved than straight, monopodially branching filaments. Spores on the sporophores are single, spherical approximately 1.8 μm in diameter and dispersing more or less evenly on hyphal filaments. Spores are either sessile or on the end of short sporophores. In broth cultures spores were not observed on the hyphae presumably because the release of mature spores is very quick.

Cultural-macromorphological properties:

Czapek-sucrose agar: Medium growth, the colonies have reddish colour covered by point-like black sporulating areas.

Glucose-asparagine agar: The growth was recorded as point like and elevated, reddish-brown or black colonies. Reddish diffusible pigments.

Nutrient agar: Fair growth, elevated, reddish-brown or black colonies. Reddish-brown exopigment in the medium.

Yeast extract-malt extract agar (ISP Med.2): Well developed, elevated and wrinkled, brown colonies, covered partly with black sporulating areas or with "pseudo-aerial mycelium" (this is appearing as a restricted whitish or greyish bloom). Brownish or brownish-red soluble pigment.

Inorganic salts-starch agar (ISP Med. 4): Medium growth of reddish-brown elevated and wrinkled colonies. Light reddish soluble pigment.

Glycerol-asparagine agar (ISP Med. 5): Growth only in traces, off-white or light orange coloured, flat coloni s, light rose soluble pigment.

Carbon source utilization: Good growth on and positive utilization of L-arabinose, D-galactose, D-fructose, D-glucose, D-xylose, lactose, melibiose, sucrose, D-mannitol, dulcitol, glycerol and inositol. Growth with L-rhamnose, D-raffinose and inulin was slightly better than on the negative control medium. Nitrogen source utilization: Good growth with yeast extract and NZ-Amine, no or weak utilization of $NaNO_3$.

Other physiological-biochemical properties: Cellulose and starch are hydrolyzed, milk is digested strongly. Nitrate reduction test is negative. No growth on potato slices without calcium carbonate (pH 5.8-6.0). No melanoid pigment production.

This strain No. IDR-P_3 of *Micromonospora* sp. was isolated from a mud sample of Lake Balaton (Hungary).

Systematic position: Further comparative systematic studies would be necessary to clarify the exact taxonomic position of this strain among the species of the genus *Micromonospora*. On the basis of certain properties it seems to be not impossible, that strain IDR-P_3 represents a new species within the genus *Micromonospora*.

Differential-diagnostic description and identification of Micromonospora strains IDR-P_4, -P_5, -P_6 and P_7

Strain IDR-P_4

On the above listed diagnostic media, generally, good growth, orange to orange red, red, sometimes yellowish or rose coloured colonies. Soluble pigments and aerial mycelium are not produced. The number of solitary spores is relatively low. They occur on the sporophores terminally. Substrate mycelium is composed of well branching hyphae. Aerial mycelium absent. No growth on D-melibiose, raffinose, mannitol, glycerol, lactose, L-rhamnose but good growth on D-arabinose, glucose, D-xylose and weak growth on D-galactose and D-fructose. On the basis of these conventional diagnostic properties we have

identified this strain as a member of species *Micromonospora purpurea* (Luedemann and Brodsky, 1964).

Strain IDR-P_5

This strain produces mostly solitary sporophores and sphaerical dark brown to black spores (0.8-1.5 µm in diameter) which adhere firmly to the sporophores until maturation. According to our electronmicroscopic observations, on the surface of these spores warty structures or outgrowths ("blunt spines" according to the Vol. 4 of Bergey's Manual of Syst. Bact. 1989, pages 2448) can be observed, which is very characteristic of the spores of *Micromonospora echinosora*. Otherwise, the cultural-morphological and physiological diagnostic properties of this strain are also very similar to those of the *M. echinospora*. The colour of the well developed colonies on the standard diagnostic media is orange-brown or dark purple. The sporulating layer is black or purplish black, waxy. Aerial mycelium absent. Melanin pigment not produced. Milk digested. Good growth on D-xylose, D-arabinose, D-glucose, and sucrose, but no growth with L-rhamnose, raffinose, D-galactose, D-fructose, D-melibiose and glycerol. We consider this strain as a typical member of *Micromonospora echinospora*.

Strain IDR-P_6

On the majority of diagnostic media moderate to weak growth. The orange or orange red colonies consist of long branched filaments (appr. 0.6 µm in diameter) and a limited number of solitary, sphaerical, dark coloured spores (0.6-1.0 µm in diameter). Does not produce aerial mycelium. In certain media weak reddish or rose coloured soluble pigments are formed. On tyrosine agar melanoid pigments were not produced. On a basal medium the following carbon sources have been utilized by this strain: D-xylose and D-fructose; only weakly: D-melibiose, mannitol and galactose, but no or sporadic growth was observed with glycerol, L-rhamnose, lactose and raffinose (see also Kawamoto, I. et al.: Agric. Biol. Chem., 47, 203-215, 1983). Strain No. IDR-P_6 shows a considerable similarity to the species *Micromonospora megalomicea*, (Weinstein, 1972) and we consider it as a member of this sp cies.

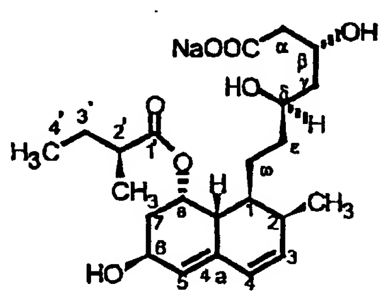
Strain IDR-P_7

Good to moderate growth on Bennett agar, Czapek sucrose agar, glucose-asparagine agar, nutrient agar, oatmeal agar, potato-dextrose agar, etc. The colour of the vegetative mycelial pigments ranges from reddish-brown to purplish-brown. On certain media wine red diffusible pigments are formed. On the surface of the colonies black spots are frequently produced. Vegetative hyphae (average diameter: 0.5 μm) are intensively branched. Spores (1.4-1.7 μm in diameter) are borne singly, sessile or on short sporophores and occur along the length of the hyphae. Growth and sporulation are of open web type of Luedemann. The following compounds are utilized by this strain as only source of carbon in medium: D-glucose, lactose, D-mannitol. L-rhamnose, sucrose and D-xylose. Dulcitol, glycerol, D-melibiose and D-raffinose are not utilized. We have identified strain No. IDR-P_7 as a typical member of *Micromonospora rosaria* (Horan and Brodsky, 1986).

The above presented *Micromonospora* strains were deposited at the National Collection of Agricultural and Industrial Microorganisms (NCAIM), Budapest, Hungary, under the below given number-designations:

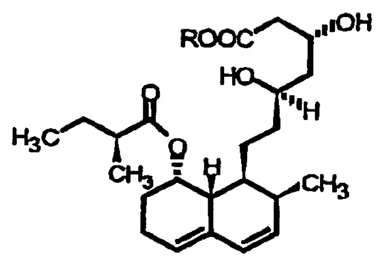
Micromonospora sp. IDR-P_3	NCAIM (P) B 001268
Micromonospora purpurea IDR-P_4	NCAIM (P) B 001271
Micromonospora echinospora ssp. echinospora IDR-P_5.	NCAIM (P) B 001272
Micromonospora megalomicea ssp. nigra IDR-P_6.	NCAIM (P) B 001273
Micromonospora rosaria IDR-P_7.	NCAIM (P) B 001274

Based on the above the invention relates to a new microbial process for the preparation of pravastatin of formula (I)



(I)

from a compound of general formula (II),



(II)

wherein R stands for an alkali metal or ammonium ion,
by the submerged cultivation of a strain which is able to 6β-hydroxylate a compound of formula (II) in aerobic fermentation and by the separation and purification of the compound of formula (I) formed in the course of the bioconversion comprising the steps of

a) cultivating a strain of a species belonging to the genus *Micromonospora* which is able to 6β-hydroxylate a compound of formula (II) - wherein R is as defined above - on a nutrient medium containing assimilable carbon- and nitrogen sources and mineral salts at 25-32°C, thereafter
b) feeding the substrate to be transformed into the developed culture, then
c) hydroxylating the substrate until the end of bioconversion, then
d) separating the compound of formula (I) from the culture broth and, if desired, purifying the same.

The scope of the invention extends to the wild strains and any mutants of species belonging to the genus *Micromonospora* which are able to hydroxylate the sodium salt of the acid form of compactin to pravastatin.

According to a preferred embodiment of the present invention pravastatin is produced with a *Micromonospora* strain selected from the group consisting of *Micromonospora sp.* IDR-P_3 [NCAIM (P) B 001268], *Micromonospora purpurea* IDR-P_4 [NCAIM (P) B 001271], *Micromonospora echinospora* IDR-P_5 [NCAIM (P) B 001272], *Micromonospora megalomicea* IDR-P_6 [NCAIM (P) B 001273] and *Micromonospora rosaria* IDR-P_7 [NCAIM (P) B 001274].

According to the most preferred embodiment of the invention pravastatin is produced with *Micromonospora* sp. strain IDR-P_3 [NCAIM (P) B 001268].

The present invention can be carried out by in situ fermentation method, that is when, hydroxylation is accomplished with the participation of an actively growing *Micromonospora* culture.

The hydroxylation may be conducted by employing agitation as shake-flask culture or aeration and agitation in fermentors, when the compound of the formula (II) is added to the growing cultures. In such cases an anti-foaming agent may be employed. The adequate density of culture of this strain could be achieved by the use of an appropriate medium containing available carbon and nitrogen sources, inorganic salts as well as trace elements.

E.g. glucose, glycerol, dextrin, starch, rhamnose, xylose, sucrose and soluble starch proved to be assimilable carbon sources while soybean meal, corn steep liquor, peptone, yeast extract, meat extract, ammonium citrate and ammonium sulfate as good nitrogen sources. Inorganic salts such as calcium carbonate, sodium phosphates, potassium phosphates etc., may be added to the culture medium. Preferred media for the growth of this selected strain are those described in the examples.

The bioconversion of compactin to pravastatin can be done by different fermentation techniques, e.g., batch culture, fed-batch culture. Preferably, an agitated liquid submerged culture is used. The preferred temperature is about 25°C to 37°C, most preferably about 25°C to 32°C.

The preferred pH is about 6.0 to 9.0, most preferably about 7.0 to 8.5. The preferred shaking condition is about 200 rpm to 400 rpm, most preferably about 250 rpm.

The invention provides a method for converting compactin acid sodium salt to pravastatin. Compactin acid sodium salt can be used in this invention at any concentration which will result in production of pravastatin. Preferably, the compactin concentration is between 0.1 and 10 g/liter, more preferably is between about 0.3 and 3.0 g/liter.

The invention is meant to cover any percentage of conversion of compactin to pravastatin by the strains of *Micromonospora* spp., at least 30% and most preferably at least about 90%.

In the course of the fermentation the composition of the culture broth is controlled by a high performance liquid chromatographic (HPLC) method. According to the HPLC method the sample of the broth is diluted twofold with methanol, centrifuged and the supernatant is used for the analysis. Parameters of the HPLC system used for the analysis are: Waters analytical HPLC equipment; column packing: Waters Novapack C_{18} 5µm; measurement at 237 nm; injection volume 10 µl; flow rate 0.6-0.9 ml/min linear gradient; gradient elution is used, eluents: solvent A = acetonitrile - 0.1M NaH_2PO_4 in water (25:75), solvent B = acetonitrile - water (pH 2 with H_3PO_4) (70:30).

Parameters of gradient elution:

Time (min)	Flow rate (ml/min)	Eluent A (%)	Eluent B (%)
0	0.6	100	0
2	0.7	100	0
12	0.9	0	100
21	0.9	0	100
22	0.9	100	0
27	0.7	100	0

Retention times: pravastatin (Na salt) 10.6 min; compactin (acid Na salt) 19.5 min; pravastatin (lactone form) 17.3 min, compactin (lactone form) 23.5 min.

Any known method can be used for the isolation of pravastatin, e.g., extraction-reextraction, anion exchange chromatography, precipitation.

For the recovery of the product from the broth it is advantageous to take into consideration the fact, that during the bioconversion pravastatin is formed in its acidic form, thus it can be isolated from the filtrate of the broth by its adsorption on an anion xchang resin column. For th isolation of the product it is advantageous to use a strongly basic anion exchange resin which is a polystyrene-divinylbenzene polymer carrying quaternary ammonium active

groups e.g. Dowex Al 400 (OH^-), Dowex 1x2 (OH^-), Dowex 2x4 (OH^-), Amberlite IRA 900 (OH^-) resins. The product adsorbed on the ion exchange resin can be eluted from the column by aqueous acetic acid or a sodium chloride containing acetone - water mixture, preferably by 1% sodium chloride containing acetone - water (1:1) mixture. Pravastatin containing fractions are combined and the acetone being in the eluate is distilled off in vacuum. The pH of the concentrate is adjusted with 15% sulphuric acid into the range of 3.5-4.0 and the acidified aqueous solution is extracted by ethyl acetate. From the ethyl acetate extract pravastatin can be extracted by 1/10 and 1/20 volume ratio of 5% sodium hydrogen carbonate or weakly alkaline water (pH 7.5-8.0). It was experienced, that pravastatin can be recovered in a pure form from the above obtained alkaline aqueous extract by column chromatography on a non-ionic adsorption resin. An advantageous method is, that first of all the ethyl acetate dissolved in the aqueous phase is removed by vacuum distillation from the alkaline aqueous extract and then the aqueous extract is loaded on a Diaion HP-20 column. Pravastatin adsorbed on the column is purified by elution with aqueous acetone in which the acetone content is gradually increased, then the chromatographic fractions containing pravastatin as a single component are combined and concentrated in vacuum. The concentrate is clarified with charcoal and lyophilized, then crystallized from an ethanol - ethyl acetate mixture, affording pravastatin in a quality acceptable for pharmaceutical application.

After finishing the bioconversion pravastatin can be extracted either from the fermentation broth or from the filtrate obtained after the separation of the micelium mass. The latter can be removed either by filtration or centrifugation, however, it is advantageous especially in an industrial scale to make a whole broth extraction. Before extraction the pH of either the fermentation broth or the filtrate of the broth is adjusted to 3.5-3.7 with a mineral acid preferably with diluted sulphuric acid. The extraction is done with acetic acid ester with a 2-4 carbon atom containing aliphatic alcohol preferably with ethyl acetate or isobutyl acetate. The ethyl acetate extract is washed with water and dried with anhydrous sodium sulphate. Then the lactone derivative is prepared from pravastatin. The lactone ring closure is carried out in dried ethyl acetate solution at room temp rature, under continuous stirring by inducing th lactone formation with catalytic amount of trifluoro-acetic acid. The lactone ring closure is checked by thin layer chromatographic analysis (TLC). After finishing the

lactone formation the ethyl acetate solution is washed at first with 5% aqueous sodium hydrogen carbonate solution and then with water, and it is dried with anhydrous sodium sulphate and evaporated in vacuum. The residue is purified with silica gel column chromatography used as the eluent mixtures of ethyl acetate - n-hexane with gradually increasing ethyl acetate content. Pravastatin is prepared from the pravastatin lactone by hydrolysis at room temperature in acetone with equivalent quantity of sodium hydroxide. When the pravastatin sodium salt formation has been completed, the pravastatin is precipitated with acetone. Then the precipitate is filtered and washed with acetone and n-hexane and dried in vacuum, then crystallized from an ethanol - ethyl acetate mixture.

It was found, that the chromatography on Sephadex LH-20 gel is advantageously applicable for purifying pravastatin. By application of this method pravastatin exceeding the purity of 99.5% (measured by HPLC) can be produced.

In the course of our experiments the following invention has been recognized: from the organic solvent extract, preferably from the ethyl acetate or isobutyl acetate extract of the broth or the broth filtrate of *Micromonospora* sp. IDR-P_3 strain which is able to 6β-hydroxylate a compound of general formula (II), pravastatin can be precipitated as a crystalline salt with secondary amines. Further it was found, that for the salt formation several secondary amines containing alkyl-, cycloalkyl-, aralkyl- or aryl-substituents are appropriate. Expediently non-toxic secondary amines were selected among them, e.g., dioctylamine, dicyclohexylamine, dibenzylamine. The isolation of the organic secondary amine salt intermediates, e.g., the dibenzylamine salt was carried out by adding dibenzylamine in 1.5 equivalent quantity related to the pravastatin content of the extract, then the extract is concentrated by vacuum distillation to 5% of its original volume, then another quantity of dibenzylamine is added into the concentrate in 0.2 equivalent ratio. The crystalline dibenzylamine salt is precipitated from the concentrate. The crystalline crude product is filtered and dried in vacuum, and it is clarified with charcoal in methanol or acetone solution. Then with recrystallization of the clarified product from acetone chromatographically pure pravastatin dibenzylamine salt intermediate can be obtained.

Pravastatin organic secondary amine salts can be transformed to pravastatin by sodium hydroxide or a sodium alkoxide preferably sodium ethoxide.

The isolation of pravastatin via a secondary amine salt intermediate is a simpler procedure than any of the ever known isolation procedures. During the procedure artefacts are not formed, and the separation of pravastatin from the by-products of the bioconversion and from the various metabolic products biosynthesized by the hydroxylating microorganism can be advantageously solved.

The process according to the invention is presented by the following examples.

Example 1

Spores were obtained from the surface of a 7-10 day old, soluble starch agar (SM) slant culture of *Micromonospora* sp. IDR-P_3 [NCAIM (P) B 001268] strain and suspended in 5 ml of sterile distilled water. This suspension was then used to inoculate 100 ml of sterile TI inoculum medium in a 500 ml Erlenmeyer flask.

Composition of SM medium

Soluble starch	10.0 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.25 g
KCl	0.2 g
$MgSO_4x7H_2O$	0.2 g
Agar	15.0 g

in 1000 ml of distilled water

The pH of the medium was adjusted to 7.0 before sterilization and the mixture was sterilized at 121°C for 25 minutes.

Composition of TI medium

Soluble starch	20.0 g
Yeast extract	10.0 g

in 1000 ml of tap water

The pH was adjusted to 7.0 before sterilization and heat treated at 121°C for 25 minutes.

The developing culture was shaken on a rotary shaker (250 r.p.m.; and amplitude: 2.5 cm) for 3 days, at 32°C, then 5 ml aliquots from it were used to inoculate 10 Erlenmeyer flasks of 500 ml volume each containing 100 ml of TT medium sterilized at 121°C for 25 minutes.

Composition of TT medium	
Potato starch	30.0 g
Soybean meal	30.0 g
$CaCO_3$	5.0 g
$CoCl_2x6H_2O$	2.0 mg
Palm oil	2.0 g
in 1000 ml of tap water	

The pH was adjusted to 7.0 before heat sterilization.

The incubation was carried out at 32°C for 72 hours then 50 mg of compactin acid sodium salt was added to each flask in distilled water, and the cultivation was carried out for 96 hours. The conversion rate of compactin acid sodium salt into pravastatin measured by HPLC was 82%.

After finishing the fermentation the cultures were united, and from the obtained collective fermentation broth, which contained 410 mg of pravastatin, the isolation of the latter was carried out as follows: The fermentation broth was centrifuged at 2500 r.p.m. for 20 min. The supernatant of the broth and the mycelial mass were separated, then the latter was resuspended in 250 ml of water and the obtained suspension was stirred for one hour and filtered. The pH of the combined centrifuged broth and the filtrate was adjusted by 15% sulphuric acid to 4.0, then the acidic filtrate was extracted with 3x300 ml of ethyl acetate. The combined ethyl acetate extracts were washed with 300 ml of water, dried with anhydrous sodium sulphate and concentrated in vacuum to 100 ml volume. Then pravastatin lactone was prepared from pravastatin by adding trifluoroacetic acid in catalytical amount at room temperature under continuous stirring. Formation of pravastatin lactone was controlled by TLC method: adsorbent: Kieselgel 60 F_{254} DC (Merck) aluminium foil; developing

solvent: acetone - benzene - acetic acid (50:50:1.5) mixture; detection: with phospho-molybdic acid reagent. The R_f value of pravastatin lactone was 0.7. After the completion of the lactone formation the ethyl acetate was washed with 2x20 ml of 5% aqueous sodium hydrogen carbonate then washed with 20 ml of water, dried with anhydrous sodium sulphate and evaporated in vacuum. 0.5 g of evaporation residue was obtained, which was chromatographed on 10 g of Kieselgel 60 adsorbent containing column (diameter of the column: 1.2 cm, height of the adsorbent bed: 17 cm). For elution ethyl acetate - n-hexane mixtures were used in which the ethyl acetate content was gradually increased. Pravastatin lactone was eluted from the column with the mixture of 60% ethyl acetate - 40% n-hexane. The fractions containing pravastatin lactone were combined and evaporated in vacuum. The residue obtained, which contained 230 mg of pravastatin lactone, was dissolved in 5 ml of acetone and then under stirring 110 mole% of sodium hydroxide was added in 1M ethanolic solution. Stirring of the solution was continued for half an hour at room temperature. Subsequently, the solution was concentrated to 2 ml volume and 4 ml of acetone was added to the concentrate. The mixture was kept at +5°C overnight. The precipitate was filtered, washed with 2 ml of acetone and then with 2 ml of n-hexane and dried in vacuum at room temperature. The resulting crude pravastatin was dissolved in ethanol, clarified by charcoal, then crystallized from ethanol - ethyl acetate mixture. In this way 170 mg of pravastatin was obtained.

Melting point 170-173°C (decomp.)

$[\alpha]_D^2$ = + 156° (c = 0,5, in water).

Ultraviolet absorption spectrum (20 µg/ml, in methanol): λ_{max} = 231, 237, 245 nm (log ε = 4.263; 4.311; 4.136).

Infrared absorption spectrum (KBr): νOH 3415, νCH 2965, νC=0 1730, νCOO^- 1575 cm^{-1}.

^{1}H-NMR spectrum (D_2O, δ, ppm): 0.86, d, 3H (2-CH_3); 5.92, dd, J = 10.0 and 5.4 Hz, 1H (3-H); 5.99, d, J = 10.0 Hz, 1H (4-H); 5.52, br, 1H (5-H); 4.24, m, 1H (6-H); 5.34, br, 1H (8-H); 4.06, m, 1H (β-H), 3.65, m, 1H (δ-H); 1.05, d, 3H (2'-CH_3); 0.82, t, 3H (4'-H_3).

^{13}C-NMR spectrum (D_2O, δ, ppm): 15.3, q (2-CH_3); 139.5, d (C-3); 129.5, d (C-4); 138.1, s (C-4a); 127.7, d (C-5); 66.6, d (C-6); 70.1, d (C-8); 182.6, s

(COO^-); 72.6, d (C-β); 73.0, d (C-δ); 182.0, s (C-1′); 18.8, q (2′-CH_3); 13.7, q (C-4′).
Positive FAB mass spectrum (characteristic ions): $[M+Na]^+$ 469; $[M+H]^+$ 447.
Negative FAB mass spectrum (characteristic ions): $[M-H]^-$ 445, $[M-Na]^-$ 423, m/z 101 [2-methyl-butyric acid-H]$^-$.

Example 2

10 Erlenmeyer flasks of 500 ml volume each containing 100 ml of MT_1 bioconversion medium were inoculated with inoculum culture prepared as described in Example 1, then incubated at 28°C for 96 hours and 50 mg of compactin acid sodium salt was added to each flask in distilled water, then the hydroxylation was carried out for 72 hours when another 50-50 mg of substrate was added to the cultures in distilled water and the fermentation was continued for 72 hours.

Composition of MT_1 bioconversion medium

Potato starch	10.0 g
Dextrose	20.0 g
Soybean meal	10.0 g
Yeast extract	10.0 g
$CaCO_3$	5.0 g
Sunflower oil	2.0 g
in 1000 ml of tap water	

The pH of the bioconversion medium was adjusted to 7.0 before sterilization. The mixture was sterilized at 121°C for 25 minutes.

After finishing the bioconversion period the cultures were united and the pravastatin was isolated from the collective broth according to the following procedure:
The united broth, which contained 750 mg of pravastatin according to the HPLC assay was centrifuged at 2500 r.p.m. for 20 min. The separated micelium mass was stirred with 250 ml of water for an hour, then filtered. The centrifuged broth

and the filtrate were combined and the pH of the resulting solution was adjusted to a 3.5-4.0 value, with 15% sulphuric acid, then the solution was extracted with 3x300 ml of ethyl acetate. Then 150 mole% of dibenzylamine-calculated for the pravastatin content - was added to the ethyl acetate extract. The ethyl acetate extract was evaporated to about 30 ml volume and the suspension was kept overnight at 0-5°C. The precipitated pravastatin acid dibenzylamine salt was filtered and washed on the filter with cooled ethyl acetate and n-hexane, finally dried in vacuum. The 1.1 g of crude pravastatin acid dibenzylamine salt was dissolved in 33 ml of acetone at 62-66°C temperature, and the solution was clarified with 0.1 g of charcoal for half an hour. Then the charcoal was removed by filtration from the solution. Crystals precipitated from the clarified solution were dissolved again at the above temperature, then the solution was kept at +5°C overnight. The precipitate was filtered, washed with cooled acetone and n-hexane and dried in vacuum. Pravastatin acid dibenzylamine salt obtained (0.7 g) was suspended in 10 ml of ethanol, then 110 mole% of sodium hydroxide was added to the solution by feeding 1M aqueous solution. Stirring of the alkaline solution was continued for half an hour at room temperature. After the completion of the sodium salt formation 30 ml of water was added and the pH of the solution was neutralized, then ethanol was distilled off in vacuum. The aqueous concentrate was chromatographed on a column filled with 50 ml of Diaion HP 20 resin (diameter of the column: 1.5 cm, height of the resin bed: 28 cm). The column was eluted with acetone-deionized water mixtures, where the concentration of the acetone was increased in 5% steps. Pravastatin could be eluted from the column by a 15% acetone containing acetone-deionized water mixture. Fractions were analysed by TLC method given in the Example 1. The R_f value of pravastatin was 0.5. Fractions containing pravastatin were combined and the acetone content was evaporated in vacuum. By the lyophilization of the aqueous residue 390 mg of chromatographically pure pravastatin was obtained.

Example 3

4.5 litres of TT/2 medium, in a laboratory fermentor, were sterilized at 121°C for 45 minutes and inoculated with 500 ml of inoculum shake culture prepared as described in Example 1, then incubated at 32°C, aerated with 250 l of sterile air/h and stirred with a flat blade stirrer at 300 r.p.m. The incubation was continued for 72 hours and 2.5 g of compactin acid sodium salt was added to the culture. After 48[th] hour of the bioconversion period the compactin substrate was completely consumed from the fermentation broth, then an additional 2.5 g of compactin acid sodium salt was added again into the culture. The second dose of compactin was consumed within 24 hours. The conversion rate of compactin acid sodium salt into pravastatin was about 90% in the bioconversion process.

Composition of TT/2 bioconversion medium

Glucose	75.0 g
Soluble starch	50.0 g
Soybean meal	50.0 g
Yeast extract	50.0 g
Pepton	5.0 g
$NaNO_3$	20.0 g
$CaCO_3$	25.0 g

in 4500 ml of tap water

Example 4

4.5 litres of the TT/1 fermentation medium, in a laboratory fermentor were sterilized at 121°C for 45 minutes and inoculated with 500 ml of the inoculum shake culture prepared as described in Example 1, then incubated at 28°C, aerated with 200 l sterile air/h and stirred with a flat blade stirrer at 400 r.p.m.

Composition of TT/1 bioconversion medium

Glucose	125.0 g
Potato starch	25.0 g
Soybean meal	50.0 g
Yeast extract (Gistex)	50.0 g
Pepton	50.0 g
$CoCl_2x6H_2O$	10.0 mg
Sunflower oil	10.0 g
in 4500 ml of tap water	

The pH of the bioconversion medium was adjusted to 7.0 before sterilization.

Cultivation was continued at 28°C for 96 hours. At this time 2.5 g of compactin acid sodium salt was added in sterile filtered aqueous solution to the culture. By the 5th day of fermentation the compactin acid sodium salt was completely consumed from the fermentation broth. Then the substrate feeding was repeated daily for further 3 days in 2.5 g/day portions. The compactin acid sodium salt substrate was gradually consumed during the four days and converted completely to pravastatin. According to the results of HPLC measurements at the end of the fermentation period from 10 g of compactin substrate 9 g of pravastatin has been produced.

After finishing the bioconversion the pravastatin formed in the concentration of 1800 µg/ml was isolated as follows:

5 litres of culture broth were centrifuged at 2500 r.p.m. for 20 min. Then 2 litres of water were added to the separated mycelial mass and the suspension was stirred for one hour and filtered. These two filtrates were united and passed through with a flow rate of 500 ml/hour on a column containing 300 g (540 ml) of Dowex Al 400 (OH^-) resin (diameter of the column: 4 cm, height of the resin bed: 43 cm), then the resin bed was washed with 1 litre of d ionized water. Thereafter the column was luted with 1 litre of acetone - water (1:1) mixture containing 10 g of sodium chloride by collecting 50 ml fractions. The fractions

were analysed by the TLC method given in the Example 1. Fractions containing the product were combined and the acetone was distilled off in vacuum. The pH of the concentrate was adjusted to 3.5-4.0 value by 15% sulphuric acid, then it was extracted 3x250 ml of ethyl acetate. 40 ml of deionized water was added to the combined ethyl acetate extract, then the pH was adjusted to 7.5-8.0 value by 1M sodium hydroxide. After 15 min stirring the aqueous and ethyl acetate phases were separated, then the ethyl acetate solution was extracted with 2x40 ml of deionized water as it was written before. Then the combined alkaline aqueous solution was concentrated to 50 ml volume and chromatographed on a column filled with 600 ml of Diaion HP20 (Mitsubishi Co., Japan) non ionic adsorbent resin (diameter of the column: 3.8 cm, height of the resin bed: 53 cm). The column was washed with 600 ml of deionized water, then eluted with acetone - deionized water mixtures, where the concentration of acetone was increased in 5% steps, collecting 50 ml fractions.The eluate was analysed by TLC method given in the Example 1. Pravastatin was eluted from the column by an acetone - deionized water mixture containing 15% of aceton. Fractions containing pravastatin as single component were combined and the solution was concentrated in vacuum to 150 ml volume. Subsequently, 0.6 g of charcoal was added to the concentrated aqueous solution and pravastatin was clarified at room temperature for 1 hour. Then the charcoal was filtered and the filtrate was lyophilised. The resulting 6.5 g of lyophilised pravastatin was crystallized twice from a mixture of ethanol and ethyl acetate. The precipitate was filtered and washed with 20 ml of ethyl acetate and 20 ml of n-hexane, and dried in vacuum at room temperature. Thus 4.6 g of chromatographically pure pravastatin was obtained.

Example 5

A spore suspension was prepared with 5 ml of sterile distilled water from the surface of a 10 days old, solubl starch agar slant culture, as described in Example 1, of *Micromonospora echinospora* ssp. echinospora IDR-P_5

[NCAIM (P) B 001272] strain - being able for the 6β-hydroxylation of compactin acid sodium salt - and the obtained spore suspension was used to inoculate 100 ml of inoculum medium TI sterilized in a 500 ml Erlenmeyer flask. Composition of the medium TI was also described in Example 1. The inoculated medium was shaken on a rotary shaker (250 r.p.m., 2.5 cm amplitude) for 3 days at 28°C, then 5 ml aliquots of the developed culture were transferred into 100-100 ml of bioconversion medium TT/1 sterilized in 500 ml Erlenmeyer flasks for 25 min at 121°C. Composition of the medium TT/1 is described in Example 4. Flasks were shaken on a rotary shaker (250 r.p.m., 2.5 cm amplitude) for 3 days at 25°C, then 10-10 mg of compactin substrate (compactin acid sodium salt) was added in sterile filtered aqueous solution into the cultures, then the fermentation was continued for 168 hours.

At the end of the bioconversion the pravastatin content of the fermentation broth was determined by an HPLC method. At this time the average pravastatin concentration was 40 μg/ml.

Example 6

The fermentation, substrate feeding and bioconversion were carried out with strain IDR-P_6, [NCAIM (P) B 001273] of *Micromonospora megalomicea ssp. nigra* as it was written in Example 5. The pravastatin content of the fermentation broth was determined by an HPLC method. At the end of the bioconversion the pravastatin content of the broth was 50 μg/ml.

Example 7

5 ml aliquots of an inoculum culture of strain IDR-P_4 [NCAIM (P) B 001271] of *Micromonospora purpurea* prepared as described in Example 1 were used to

seed 100-100 ml of TT/14 medium dispensed in 500 ml Erlenmeyer flasks and sterilezed for 25 min at 121°C.

<u>Composition of medium TT/14</u>

Potato starch	5.0 g
Glucose	25.0 g
Yeast extract (GISTEX)	15.0 g
Pepton	15.0 g
$CaCO_3$	1.0 g

in 1000 ml of tap water

The pH of the bioconversion medium was adjusted to 7.0 before sterilization.

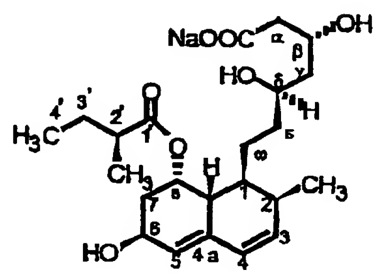
Flasks were shaken on a rotary shaker (250 r.p.m., 2.5 cm amplitude) for 3 days. The substrate feeding, the bioconversion and determination of the pravastatin content were carried out as described in Example 5. At the end of the bioconversion the pravastatin content of the fermentation broth was 40 μg/ml.

Example 8

The fermentation, substrate feeding and bioconversion were carried out with strain IDR-P_7, [NCAIM (P) B 001274] of *Micromonospora rosaria* as it was written in Example 1. At the end of the bioconversion 350 μg/ml pravastatin was measured in the fermentation broth by HPLC method.

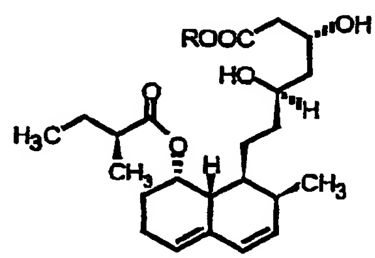
What we claim is:

1. A microbial process for the preparation of the compound of formula (I)



(I)

from a compound of the general formula (II)



(II)

wherein R stands for an alkali metal or ammonium ion,
by the submerged culture of a *Micromonospora* strain which is able to 6β-hydroxylate a compound of formula (II) under aerobic conditions and by the separation and purification of the compound of formula (I) formed in the course of the bioconversion comprising the steps of

a) cultivating a *Micromonospora* strain which is able to 6β-hydroxylate a compound of formula (II) - wherein R is as defined above - at 25-32°C on a nutrient medium containing available carbon- and nitrogen sources and mineral salts, thereafter

b) feeding the substrate to be transformed into a developing culture, then
c) hydroxylating the substrate until finishing of the bioconversion, then
d) separating the compound of formula (I) from the culture broth and, if desired, purifying the same.

2. A process as claimed in Claim 1, wherein the *Micromonospora sp. IDR-P_3* strain deposited at the National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary under the number NCAIM (P) B 001268 or a mutant strain thereof which is able to 6β-hydroxylate a compound of general formula (II) is applied.

3. A process as claimed in Claim 1, wherein the *Micromonospora purpurea IDR-P_4* strain deposited at the National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary under the number NCAIM (P) B 001271 or a mutant strain thereof which is able to 6β-hydroxylate a compound of general formula (II) is applied.

4. A process as claimed in Claim 1, wherein the *Micromonospora echinospora ssp. echinospora IDR-P_5* strain deposited at the National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary under the number NCAIM (P) B 001272 or a mutant strain thereof which is able to 6β-hydroxylate a compound of general formula (II) is applied.

5. A process as claimed in Claim 1, wherein the *Micromonospora megalomicea ssp. nigra IDR-P_6* strain deposited at the National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary under the number NCAIM (P) B 001273 or a mutant strain thereof which is able to 6β-hydroxylate a compound of general formula (II) is applied.

6. A process as claimed in Claim 1, wherein the *Micromonospora rosaria IDR-P_7* strain deposited at the National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary under the number NCAIM (P) B 001274 or a mutant strain thereof which is able to 6β-hydroxylate a compound of general formula (II) is applied.

7. A process as claimed in anyone of Claim 1 to 6, wherein the compound of formula (I) formed during the fermentation is separated from the culture broth by adsorption on an anionic ion exchange resin or by extraction with a water immiscible organic solvent, followed by the preparation of its lactone derivative or its secondary amine salt as an intermediate, or by purification of the alkaline aqueous extract obtained from the organic solvent extract of the fermentation broth with chromatography on a non-ionic adsorbing resin.

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/HU 00/00066

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12P17/06 C12P7/42

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, CHEM ABS Data, MEDLINE, CANCERLIT, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 40863 A (MASSACHUSETTS INST TECHNOLOGY) 19 December 1996 (1996-12-19) page 3, line 20 -page 10, line 28	1,7
A	US 5 179 013 A (MATSUOKA TATSUJI ET AL) 12 January 1993 (1993-01-12) cited in the application the whole document	1,7

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

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"P" document published prior to the international filing date but later than the priority date claimed

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 October 2000	13/10/2000

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INTERNATIONAL SEARCH REPORT

..formation on patent family members

Inter: nal Application No
PCT/HU 00/00066

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